UNIVERSITY OF ALBERTA

Development of Solid Phase Immunoextraction Matrix-Assisted Laser/Desorption Time-of-Flight Mass Spectrometry for Improving Analysis Efficiency of Sulfonamide Residues in Environmental Samples

BY

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
BM Assay	Bratton Marshall assay
B	absorbance of a sample in competitive ELISA
Bo	absorbance of PBST blank in competitive ELISA
BSA	bovine a serum albumin
CDR	complementarity-determining region
^{14}C	carbon 14 labelled isotope
DHB	2,5-dihydroxybenenzoic acid
DMSO	dimethylsulfoxide
EDC	1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
GAR-HRP	goat anti-rabbit antibodies conjugated to horseradish peroxidase
GC	gas chromatography
HLB	hydrophobic-lipophylic balance extraction support (Waters ^{TM})
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HZ	hydrazine
IAC	immunoaffinity chromatography
IgG	immunoglobin G
IC_{50}	analyte inhibition concentration required to achieve B = Bo/2
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
LPH	*Limulus polyphemus* Hemolymph
IS	internal standard
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MIP	molecular imprinted polymer
MRL	maximum residue limit
MS	mass spectrometry
MSPD	matrix solid phase dispersion
m/z	mass-to-charge ratio
NAHis	N-α-acetyl-L-histidine
NAHis	N-chloroacetyl-L-tyrosine
NAHis-SMT	N-α-Acetyl-L-histidine sulfamethazine
NAHis-STZ	N-α-Acetyl-L-histidine sulfthiazole
NCATyr-SMT	N-chloroacetyl-L-tyrosine sulfamethazine
NCATyr-STZ	N-chloroacetyl-L-tyrosine sulfathiazole
NA-SMT	N^4-acetylsulfamethazine
NA-STZ	N^4-acetylsulfathiazole
NOAEL	no-observed-adverse-effect-level
NP-SMT	N^4-propylsulfamethazine

NP-STZ	N^4-propylsulfathiazole
NED	N-1[napthyl]ethylenediamine dihydrochloride
NMR	nuclear magnetic resonance
PABA	*p*-aminobenzoic acid
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.05% Tween 20
ppb	parts per billion
ppm	parts per million
RT	room temperature
SD	standard deviation
SDA	sulfadiazine
SDM	sulfadimethoxine
SEM	standard error of the mean
SF	sulfanilamide
SMR	sulfamerazine
SMT	sulfamethazine
SMT-azo-BSA	azo-linked, sulfamethazine-bovine serum albumin conjugate
SMT-azo-LPH	azo-linked, sulfamethazine-*Limulus polyphemus* hemolymph conjugate
SMT-succinyl-BSA	hemisuccinate-linked, sulfamethazine-bovine serum albumin conjugate
SMT-succinyl-LPH	hemisuccinate-linked, sulfamethazine-*Limulus polyphemus* hemolymph conjugate
SPD	sulfapyradine
SPIE	solid phase immunoextraction
SPIE MALDI-TOF	solid phase immunoextraction coupled to MALDI-TOF MS
STZ	sulfathiazole, free acid
STZ-azo-BSA	azo-linked, sulfathiazole-bovine serum albumin conjugate
STZ-azo-LPH	azo-linked, sulfathiazole-*Limulus polyphemus* hemolymph conjugate
STZ-succinyl-BSA	hemisuccinate-linked, sulfathiazole-bovine serum albumin conjugate
STZ-succinyl-LPH	hemisuccinate-linked, sulfathiazole-*Limulus polyphemus* hemolymph conjugate
succinyl-SMT	N^4-succinylsulfamethazine
succinyl-STZ	N^4-succinylsulfathiazole
ScFv	single chain variable antibody fragment
TMB	3,3',5,5'-tetramethylbenzidine
TLC	thin layer chromatography
TOF	time-of-flight
TRIS	tris(hydroxymethyl)aminomethane
UV	ultraviolet
V_H	variable heavy chain antibody protein

Chapter 1

INTRODUCTION
AND
LITERATURE REVIEW

1.1. BACKGROUND

Over eons human culture evolved from a nomadic to a more stable agrarian way of life. And yet, in a relatively brief span of time, the scientific revolution has transformed civilization into a densely populated and technologically-sophisticated global community. Society now identifies with and even seeks rapid change instead of stability, where new technologies introduced to one generation are obsolete before the next, where new innovations have fundamental, encompassing, and unpredictable impacts on how people live their lives. Despite this extraordinary achievement - where physical comfort and material wealth are available to most of those who live within the "developed world" - attaining this modern reality has had many costs. Ironically, many technologies and activities responsible for these successes have burdened both the environment and civilization with potentially harmful compounds. A growing quantity and variety of chemicals are produced and used world-wide by individuals, industry, and agriculture, and reach the ecosystem with mostly unknown long-term fates and consequences. At first, these compounds were assumed to be innocuous due to the huge dilution ostensibly afforded by a seemingly limitless amount of air, land, and water. However, their ecological, economic, and social impacts are now the ongoing subjects of a great political and scientific controversy. Over the last 50 years, the immense complexity of this anthropogenic pollution dilemma is being realized, as are the potential large-scale risks to the well-being of people and other life on Earth.

Dioxin-like chemicals have been the focus of much scientific inquiry and the need to measure them in the environment spurred technological advances in residue analysis. Many of these pollutants have since been shown to be potentially harmful, yet their actual significance and impact are inconclusive and part of an on-going, controversial debate. There is growing evidence these compounds may have hormonal activity at very low concentrations, and act as hormone disrupters with a wide variety of uncertain implications to humans, agriculture, and wildlife, a problem that is compounded by their persistence (Behnisch et al., 2001; Borgeest et al., 2002; Eertmans et al., 2003; Foster, 2001; Holland, 2003; Mayani et al., 1997; Mukerjee, 1998; Nicolopoulou-Stamati and Pitsos, 2001; Ohtake et al., 2003; Pocar et al., 2003; Vos et al., 2000).

Also of concern are synthetic compounds that have, by design, a high biological activity, such as pesticides and pharmaceuticals. Reviews conducted on the environmental fate of agricultural pesticides document a large body of research over the last twenty years (Carpy et al., 2000; D'Amato et al., 2002; Pehkonen and Zhang, 2002; Petit et al., 1995; Ragnarsdottir, 2000; Stangroom et al., 2000b; Stangroom et al., 2000a; Voccia et al., 1999; Warren et al., 2003). In contrast, research and regulatory concern regarding the fate and impact of pharmaceutical contaminants in the environment is relatively new and rare (Daughton and Ternes, 1999; Drewes et al., 2002; Heberer, 2002a; Hirsch et al., 1999; Kolpin et al., 2002).

The ability to create scientific models capable of predicting the fate and impact of organic pollutants, and ultimately, enabling society to safely design and regulate compounds destined for the environment, relies upon accurate and sensitive systems of analysis. Due to the enormous number of substances and potential complexity of interactions, it is not likely that a substance-specific chemical approach alone can predict the toxicity of foods or environmental effluents, regardless of how efficient monitoring systems become (Tonkes, 2001). Rapidly evolving biochemical and bioassay methods offer many advantages in toxicity screening and risk assessment, including being less expensive, faster, very sensitive, and capable of the integrated measurement of biological activity rather than concentration alone. Despite the benefits of indirect bioassay systems of analysis, parallel chemical speciation will always be required to identify, understand and confirm modes of action of particular pollutants (Holland, 2003). Therefore, toxicity risk assessment needs to be coordinated and correlated with accurate and efficient chemical analysis systems.

Specific "bottle-necks" have greatly limited the speed of chemical analysis. Most standard methods for chemical residue testing described by the Official Methods of Analysis of AOAC International (2003) - usually chromatographic separation coupled with mass spectrometry - are slow and expensive, and require a high degree of technical expertise to operate. The nature of this investigation was motivated by the need to improve efficiency of chemical analysis systems for anthropogenic organic pollutants without compromising assay sensitivity or accuracy. In this project, sulfamethazine, an antibacterial sulfonamide compound, was selected as the drug model used for

development of a novel residue analysis system. It has the potential to be an environmental persistent pollutant, because it is both biologically active and chemically stable. It is an excellent example of a pharmaceutical which has been overlooked in the analytical and agrochemical literature. Sulfonamides represent the first organic chemotherapeutic agents synthesized to treat bacterial disease, and continue to be used in human medicine and prophilactically in animal husbandry. Most drug residue methodologies have been directed toward food animal tissues, with only a minor focus on techniques and trends in environmental analysis. Although the techniques described in this thesis could readily be adapted to food analysis, the objective encompassed a broader scope, examining sulfonamide fate within the agricultural environment, where the greatest balance of total drug mass would exist outside the animal.

1.2. GENERAL THESIS ORGANIZATION

From a wide variety of perspectives, this first chapter reviews the literature on issues pertinent to sulfonamide antibacterial compounds. Sulfonamide chemistry, biological activity, pharmacokinetics, and food and environmental residue perspectives are addressed. Contemporary sulfonamide analysis is described, and then relevant and novel elements of the current research are discussed, namely immunoaffinity chromatography of "small" (sulfonamide-size) compounds, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). At the end of this introductory chapter, the thesis objectives are outlined. The references cited, in the introduction and throughout the body of the thesis, are presented in alphabetical order in Chapter 8.

Next, the experimental materials and methods are presented, in detail, in Chapter 2. In Chapter 3, the development and characterization of ten anti-sulfonamide polyclonal antibodies are reported. Immunoaffinity columns (IACs) capable of extracting sulfonamides were made from anti-sulfonamide polyclonal antibodies, and their performances are then described in Chapter 4. A potential anti-hapten antibody screening system for the selection of IAC candidates was also demonstrated in Chapter 4, by relating IAC performance results back to the antibody characterizations of Chapter 3. This comparison was necessary to demonstrate how simple screening of polyclonal

antibody sera can be useful in predicting the ultimate IAC utility and performance. Chapters 5 and 6 document the development and application, respectively, of an analysis procedure capable of detecting trace residue (ng/L) levels of sulfonamides in environmental samples. This solid phase immunoextraction (SPIE) MALDI-TOF MS method was tested both by recovering and detecting fortified sulfonamides in water, soil, and manure samples (Chapter 5), and later, by detecting sulfonamide residues in farm site water, soil, and manure samples incurred by normal agricultural practices (Chapter 6). Chapter 7 summarizes the research conducted as a whole, and then examines the implications of residue analysis technology both technologically and philosophically.

This thesis is presented in a hybrid fashion due to the large amount and of concepts and information. It is part traditional format (i.e. introduction and literature review followed by experimental, then results and discussion, then conclusions and summary, then references) and part research paper format (i.e. each chapter is self-contained with an introduction, results and discussion, conclusions and summary, and references). A hybrid format was thought to be more appropriate to effectively present the development of the ideas and methodology by presenting extra information to the reader where necessary, without over-burdening each chapter with all the redundant details of the methodologies. Although there is a detailed Experimental Methods chapter at the beginning (Chapter 2), the salient procedures and features of each experiment are briefly described in each chapter, where necessary, to bring better clarity and context to each experiment. Detailed methodology, experimental formats, equations and calculations are cross referenced in the chapters back to the Experimental Methods of Chapter 2.

In order to maintain an uninterrupted flow of ideas without over-emphasizing relevant subtleties and complexities, comments that are tangential to the concepts being discussed are related as footnotes within the chapters. Also, an appendix at the end of the document (Chapter 9) contains are large amount of experimental results and discussion regarding the preliminary development of antibodies and immunoaffinity columns, where the interested reader can examine these concepts in more detail. The appendix information is not necessary to understand the research presented in the body of the thesis,

however, it may give one a greater ability to compare and understand this research to other work that advances the field of immunological recognition of small molecules.

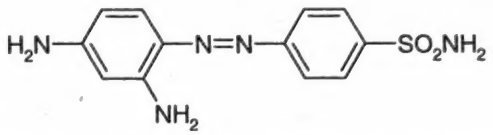
1.3. SULFONAMIDES

1.3.1. History of Sulfonamides

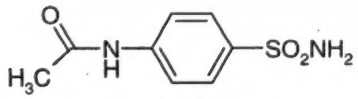
In the 1930s, the synthesis and application of sulfonamides as antibacterial agents in medicine, together with the discovery of penicillin, symbolized the breaking of a technological barrier for humanity in the advancement of chemotherapeutic treatment of disease. The story of sulfonamides starts at the beginning of the 20th century, and is summarized from an encyclopaedic series entitled Antibiotics and Chemotherapy (Vree and Hekster, 1987) and from records of the Nobel Foundation (2003). Gerhard Domagk was a medical student when World War I began. As he fought in the trenches he witnessed the huge loss of human life associated with infection secondary to battlefield injury or the vain attempts of surgery. In 1932, as research director of I.G. Farben Industrie in Germany, he discovered that Prontosil (Figure 1.1) - a synthetic dye made by the company - could protect laboratory animals against bacterial infections. In 1939, Domagk was awarded the Nobel Prize in medicine for his work with Prontosil. Alexander Fleming had discovered penicillin in 1928, and he, along with Ernst Chain and Howard Florey, were awarded the Nobel Prize for their research into the curative effects of penicillin on bacterial diseases. It was the beginning of a new era that revolutionized humanity's relationship to disease.

The activity of Prontosil was confusing at first. Animal experiments determined Prontosil was active *in vivo*, yet had no *in vitro* activity. Prontosil was a derivative of sulfanilamide (*p*-aminobenzene sulfonamide), first synthesized by Paul Glemo in 1908 for his doctoral thesis, in developing azo dyes containing the sulfonamide group (Figure 1.1). Although others in the following decade would observe some degree of antibacterial activity associated with sulfanilamide, it was not until 1935 that Jacques Trefouel, of the Pasteur Institute, would propose that Prontosil (4-(2,4-Diamino-phenylazo)-benzenesulfonamide) was being metabolized into sulphanilamide, and then this compound was excreted in the urine as acetylsulfanilamide. Albert Fuller of London then provided more definitive analytical evidence that sulfanilamide was present in the

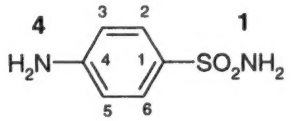
urine and blood after dosing with Prontosil. Sulfanilamide was shown to have antibacterial activity identical to Prontosil, but was also able to work *in vitro*. Many pharmaceutical companies began active research in making new derivatives and bringing



Prontosil (4-(2,4-Diamino-phenylazo)-benzenesulfonamide)



acetylsulfanilamide



sulfanilamide

Figure 1.1. Structures of Prontosil, acetylsulfanilamide, and sulfanilamide.

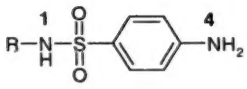
them to market, since the original patent by the inventors of Prontosil did not include sulfanilamide, now recognized to be the active form.

Myriad derivatives were synthesized by chemists (estimated to be about 5500 by the year 1945), which resulted in a battery of compounds less toxic than sulfanilamide, and with a range of different clinical applications. About 25 of these "sulfonamides", as they were called, are still in use today, and all have in common the same sulfonamide bond associated with the core structure of *p*-aminobenzene sulfonamide.

1.3.2. Chemistry of Sulfonamides

The nomenclature of sulfonamides is such that substituents of the sulfonamide nitrogen are called N^1 substituents, and substituents on the *p*-aminobenzene nitrogen are called N^4 substituents. All sulfonamides with antibacterial activity are N^1 substituents

Basic sulfonamide structure



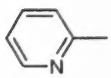
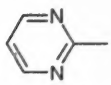
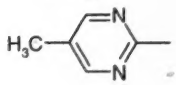
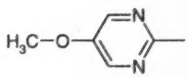
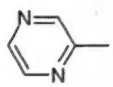
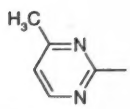
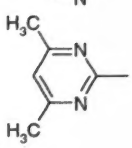
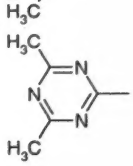
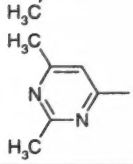
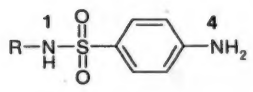
R group	Common name	pK_a of N^4 proton	Year introduced
H—	Sulfanilamide	10.7	1908
	Sulfacetamide		1939
	Sulfaguanidine	-	1940
	Sulfapyridine	8.4	1938
	Sulfadiazine	6.4	1939
	Sulfaperine	6.8	1959
	Sulfamethoxydiazine	7.0	1960
	Sulfapyrazine	-	-
	Sulfamerazine	7.1	1940
	Sulfamethazine	7.4	1942
	Sulfasymazine	-	1966
	Sulf(a)isomidine	7.4	1939

Figure 1.2. Structures of common sulfonamides (continued on following page).

Basic sulfonamide structure



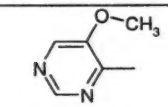
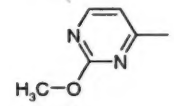
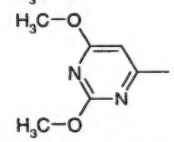
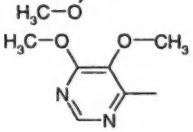
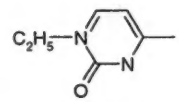
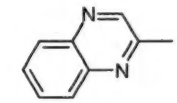
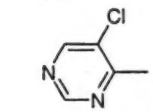
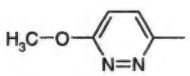
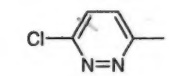
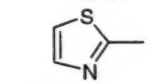
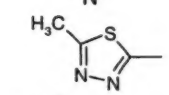
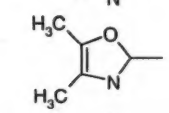
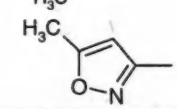
R group	Common name	pK_a of N^4 proton	Year introduced
	Sulfalene	6.3	1961
	Sulfamonomethoxine	-	1960
	Sulfadimethoxine	7.0	1947
	Sulfadoxine	6.3	1965
	Sulfacytine	6.9	1968
	Sulfaquinoxaline	6.0	1944
	Sulfachloropyridazine	-	1955
	Sulfamethoxypyridazine	7.0	1956
	Sulfaclomide	-	1966
	Sulfathiazole	7.2	1947
	Sulfamethizole	5.4	1940
	Sulfamoxole	7.2	1957
	Sulfamethoxazole	5.7	1962

Figure 1.2. (...continued) **Structures of common sulfonamides.**

which usually are five or six member heterocyclic rings (Figure 1.2). N^4 substituents have no antibacterial activity unless the substituent is hydrolyzed (Vree and Hekster, 1987).

Sulfonamides are yellowish powders, and the free acid forms are relatively insoluble in water and sparingly soluble in ethanol or acetone, whereas their sodium salts have considerably greater water solubility (Horwitz, 1981b). They are chemically stable compounds, with very slow rates of decomposition at the sulfonamide bond. Sulfathiazole (Figure 1.2) was estimated to have a half-life of 530 years at pH 3.5 and 80 °C (Pawelczyk and Zajac, 1976), and it is reasonable to estimate that most other sulfonamides structurally similar to sulfathiazole would have similar chemical stability. Sulfonamides' unique acid-base chemistry confers their characteristic pH-dependent solubility, antibacterial activity, and many of their specific pharmacokinetic properties. The primary aromatic amine of sulfonamides is weakly basic and only protonated under very acidic conditions (pK_a of aryl amines are 2 – 3). This makes the sulfonamide aromatic amine uncharged in urine, blood or most environmental samples. In sulfonamides made with heterocyclic ring N^1 substitution, the single proton of the N^1 nitrogen is weakly acidic (pK_a around 7) at physiologic pH. The heterocyclic ring is able to resonance stabilize a negative charge on the N^1 nitrogen, making it considerably more acidic compared to the unsubstituted sulfanilamide (Figure 1.3). Sulfonamides are generally much more soluble in neutral pH or basic conditions because they become predominantly negatively charged. At slightly acidic conditions they are much less soluble, effectively becoming neutrally charged at their isoelectric point (Vree and Hekster, 1987), which is an important fact considered later in their determining their extraction during analysis, their renal toxicities, or their environmental fates.

1.3.3. Pharmacology of Sulfonamides

Mode of Action of Sulfonamides

Sulfonamides are bacteriostatic to Gram-positive and Gram-negative bacteria, and the developmental history of their mode of action is well documented (Anand, 1975). Any N^4 substitutions, such as enzymatic acetylation by the animal liver, eliminate the bacteriostatic activity of a sulfonamide. They are structurally very similar to *p*-aminobenzoic acid (PABA) (Figure 1.4), and are competitive antagonists of PABA by

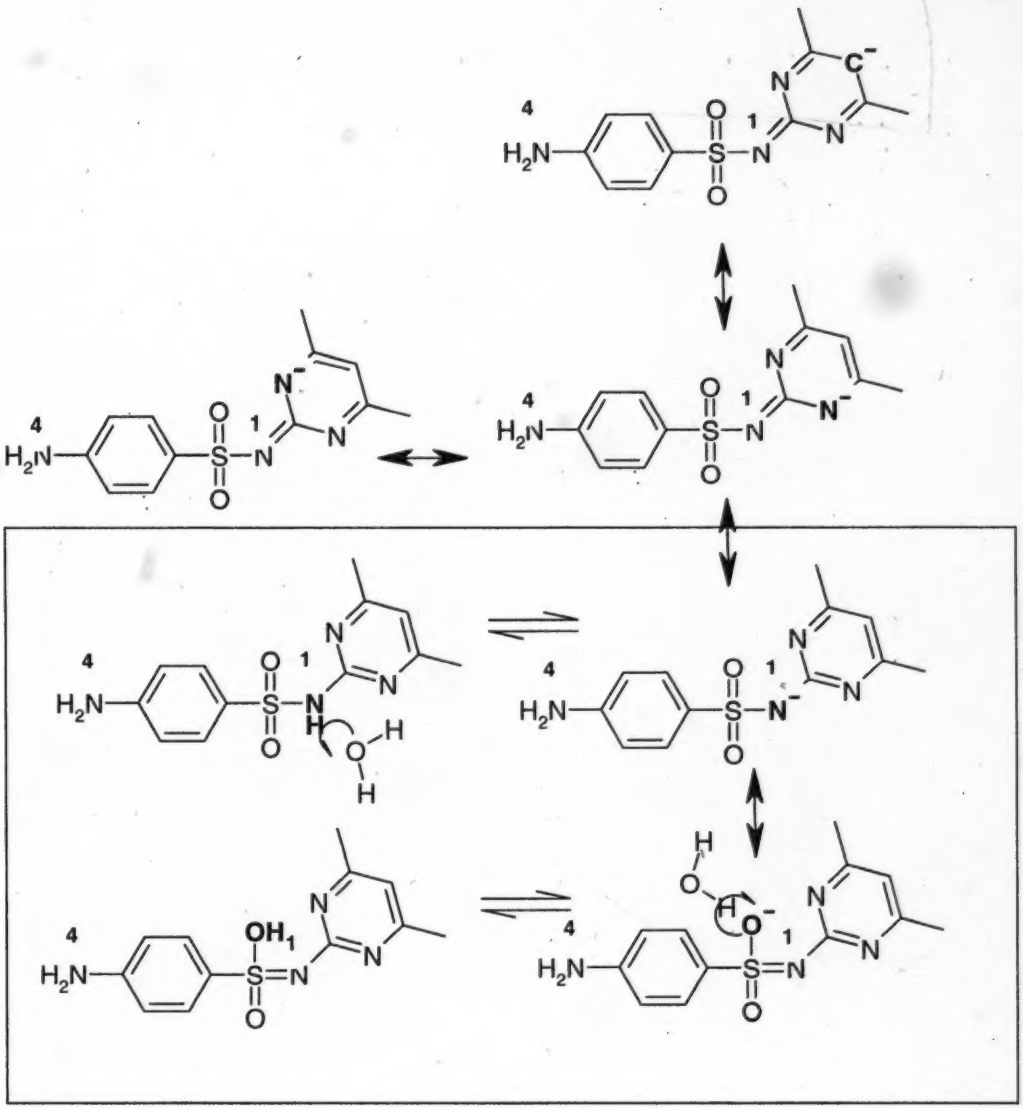


Figure 1.3. Various forms of sulfamethazine. The major ionic and resonant states of sulfamethazine at physiological pH are enclosed by the box.

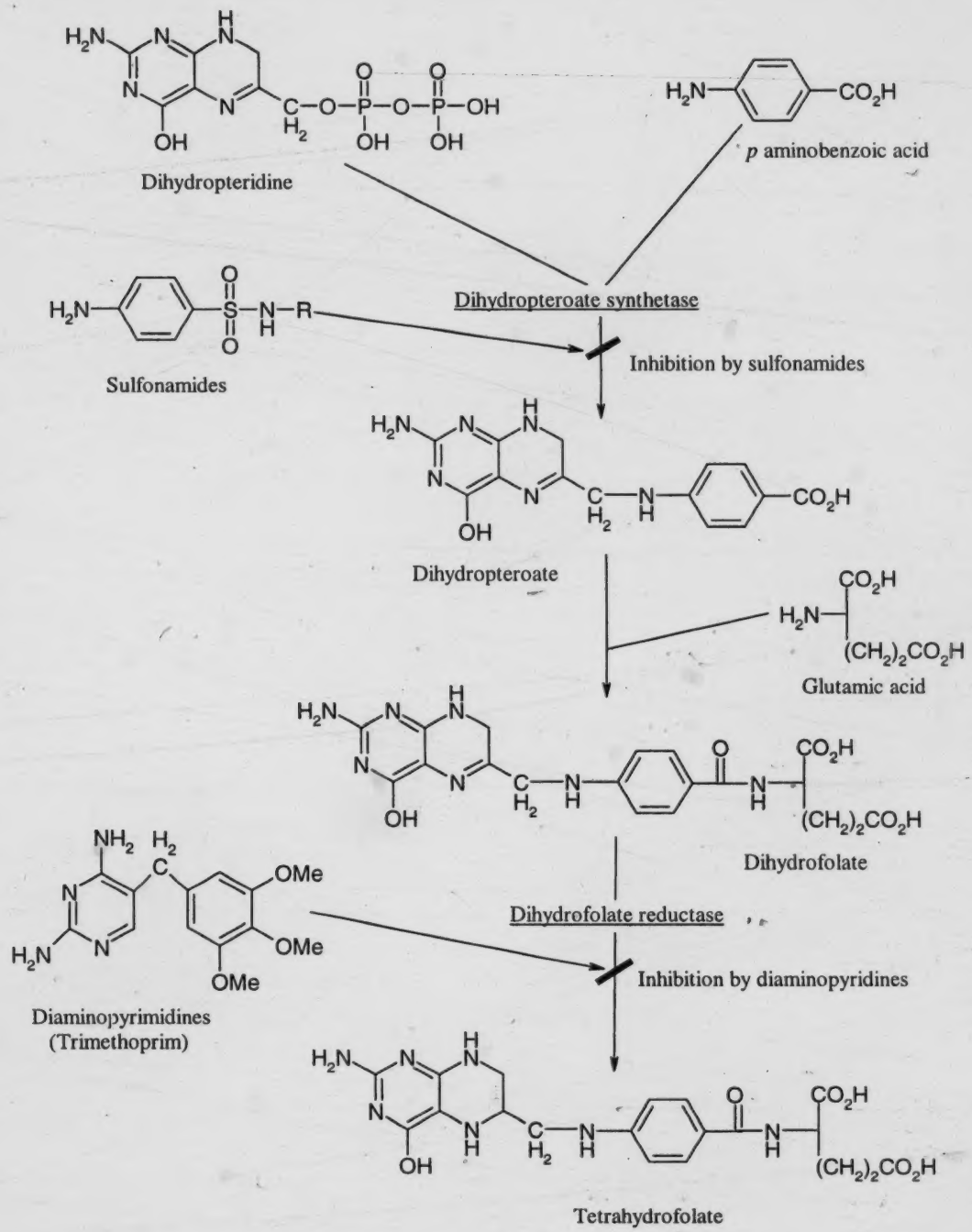


Figure 1.4. Enzymatic synthesis of tetrahydrofolate (Adapted from Anand, 1975).

inhibiting dihydropteroate synthetase which requires PABA as a precursor in the synthesis of tetrahydrofolate.

Tetrahydrofolate is an important intermediate metabolite for many one-carbon transfer reactions in the synthesis of some amino acids and nucleotides, so inhibition of tetrahydrofolate synthesis causes bacteria to stop growing and reproducing. It has been shown that sulfonamides with higher electronegativity at the SO_2 group have higher bacteriostatic activity (Bell and Roblin, 1942), which is logical since in an ionized state sulfonamides would more closely resemble the physiological ionic state of the PABA precursor (Soriano-Correa et al., 2003). However, a sulfonamide's bacteriostatic activity is also dependent on its uncharged, hydrophobic character, conferring its ability to transport across cellular membranes. Thus, a balance between a sulfonamide's ionized state and its hydrophobic nature are achieved by designing sulfonamides possessing pK_a values near physiological pH, thus allowing for both its cellular distribution and ultimate competition with PABA at the enzyme level (Anand, 1975; Mengelers et al., 1997). Sulfonamides administered to animals specifically affect bacteria, since animals do not synthesize folic acid. Animals require preformed folic acid in their diets, which is usually unavailable for bacteria because it is conjugated to polyglutamate and proteins.

Pharmacokinetics of Sulfonamides

Sulfonamides can be administered by oral, intravenous, intramuscular, intraperitoneal, intrauterine, and topical routes. Oral and topical routes are most common in human medicine, whereas oral and injectable routes are most commonly used in veterinary medicine (Bevill, 1984). They are absorbed through the small intestine except for a few that are poorly absorbed and designed to treat bowel infections (e.g. sulfaguanidine). After absorption, sulfonamides and their metabolites are widely distributed throughout the body. They can be detected in the urine, saliva, sweat, bile, breast milk, cerebrospinal fluid, peritoneal, ocular and synovial fluids, as well as pleura and other effusions (Martindale, 1982). In the blood, sulfonamides are predominantly bound to albumin protein (Mandell and Sande, 1990). This reversible protein binding is important because only the unbound fraction possesses an antibacterial effect. Although the metabolism and excretion of sulfonamides varies with species, in general, more than 90% of sulfonamides are eliminated by renal excretion, and the main metabolite is

usually the N^4-acetyl derivative. Depending on the sulfonamide and the species, the half-life of a sulfonamide can vary dramatically. For example, for the short-acting sulfamethazine, the half-life is about 1.5-5.5 h in humans, 9-25 h in cows, and 6 h in rats, whereas for the longer-acting sulfadimethoxine (Figure 1.2) in the same animals it is 35, 17, and 28 h, respectively (Vree and Hekster, 1985). The half-life of sulfamethazine in the pig is 13 h, and 90% of the drug in plasma is represented by the parent compound, with most of the remaining 10% existing as metabolites (Nouws et al., 1986b). Over 50% of excreted sulfamethazine in pigs is in the N^4-acetyl form, which has relatively high renal clearance rates. Other forms excreted in the urine in relatively equal amounts include the parent sulfonamide, glucuronide and glucoside metabolites, and various hydroxyl metabolites (Mitchell et al., 1986; Nouws et al., 1986a). Desaminosulfamethazine has been detected in pork tissues at low levels (1.2% in plasma) 24 h after feeding pigs sulfamethazine and was correlated to nitrite in the diet (Paulson and Struble, 1980). Many other sulfonamide metabolites in pigs are possible as demonstrated by research for other species and other sulfonamides (Rehm et al., 1986) (Figure 1.5). The N^4-acetyl form of sulfonamides are microbiologically inactive, whereas the hydroxylated metabolites retain some of their activity in relation to the parent drug (Nouws et al., 1985).

Adverse Reactions to Sulfonamides

The overall known incidence of adverse drug reactions from sulfonamides is about 5%, classified as either toxicological, carcinogenic, or hypersensitivity-type reactions, and are usually stopped or reversed with the cessation of sulfonamide administration (Mandell and Sande, 1990). Sulfonamides have low solubility, so they tend to crystallize in the renal tubules of the kidney (crystalluria), especially for carnivores with acidic urine near the isoelectric point of a sulfonamide, or during periods of insufficient water intake causing sulfonamide to precipitate in urine. The consequences of crystalluria are lumbar pain, hematuria (blood in the urine), and decreased kidney function. To prevent crystalluria when sulfonamides are administered, treatment with sodium bicarbonate to increase urinary pH and adequate water intake are recommended (Bevill, 1984; Rehm et al., 1986; Vree and Hekster, 1985).

Sulfamethazine was found to induce reversible hyperplasia (unusual increase in

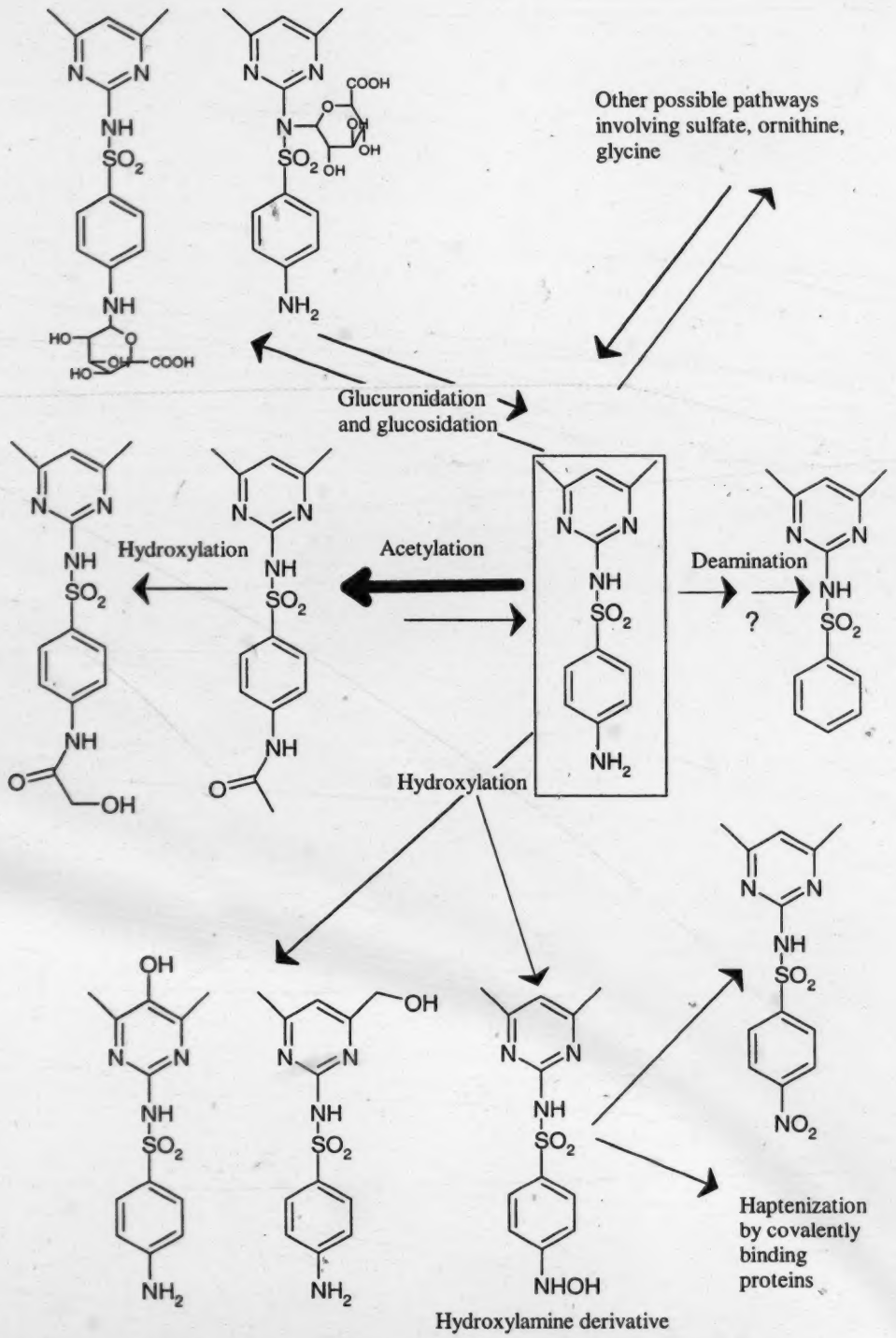


Figure 1.5. Metabolites of sulfamethazine in the pig. (Sulfamethazine is shown in the box in its unmetabolized form)

cell growth) and glandular tumours in rat thyroid (Littlefield et al., 1990), a finding that was debated as to its validity and relevance to it safety in humans (Shaw et al., 1991; Woodward, 1992). Expert panels finally concluded that high dose sulfonamide-induced hyperplasia in the rat model was not due to classical carcinogenic mechanisms, but rather to sulfonamide's high-dose goitrogenic activity (Doerge and Decker, 1994). Furthermore, they concluded that the rat model was not applicable to humans; people were not as sensitive to the sulfamethazine-like inhibition of thyroid function (Poirier et al., 1999b).

Sulfonamides' effects on the hematopoieic system include hemolytic anemia (premature destruction of red blood cells), agranulocytosis (severe reduction of granulocytes), aplastic anemia (deficiency of formed elements in the blood), thrombocytopenia (reduction of platelets), and eosinophilia (increased number of eosinophils in the blood) (Huber, 1986). The mechanisms of these hematopoieic disorders have not been clearly determined, but may be a combination of direct toxic effects on the cellular components or immunological sensitization (Mandell and Sande, 1990).

The incidence of sulfonamide hypersensitivity reactions are primarily noted in people or animals receiving them therapeutically. A therapeutic dose induces an adverse skin or upper gastrointestinal reaction in 3.5% of people (Rehm et al., 1986), and 20% demonstrate hypersensitivity if they have received previous sulfonamide therapy (Lloyd and Mercer, 1984). Stevens-Johnson syndrome - a generalized inflammatory disease affecting children and young adults - is the most serious manifestation of sulfonamide hypersensitivity and can be fatal (Anand, 1975). Adverse idiosyncratic reactions, such as "drug fever", hepatotoxicity, skin rash, blood dyscrasias (blood or bone marrow abnormalities), nephritis, and cardiotoxicity are thought to occur due to conversion of sulfonamides to reactive metabolites like hydroxylamines (Cribb et al., 1990; Cribb and Spielberg, 1990; Shear et al., 1986; Trepanier, 1999). Hydroxyamine derivatives are very labile compounds, and may bind to cellular proteins, and therefore these conjugates are large enough to initiate an allergic immune response (Figure 1.5). Thus, individuals with slow acetylator phenotypes are more predisposed to the formation of sulfonamide hydroxylamine derivatives since sulfonamides are not eliminated quickly by the normal acetylation route (Vree and Hekster, 1987).

1.3.4. The Use of Sulfonamides in Animal Husbandry and Aquaculture

Sulfonamides are used therapeutically and prophilactically in animal husbandry and aquaculture. Although their use has decreased due to the wide variety of antibiotic available in human medicine, they are still used widely to treat a range of infections in poultry, cattle, sheep, pigs, and fish due to their chemical stability if feed and water (Steele and Beran, 1984). Specific production rates of antibiotics for North American livestock are not reported in the literature, however, the European Union is estimated to consume almost 4,000 metric tonnes of therapeutics in veterinary medicine and livestock feed, 78 metric tonnes of which are sulfonamides (Thiele-Bruhn, 2003). Given to animals in feed and water, sulfonamides have been an effective sub-therapeutic means of disease prevention and in promoting the growth and efficient conversion of feed in animals (Franco et al., 1990). Through a variety of mechanisms, the classic "antibiotic growth effect" is associated with a reduction or changed intestinal microfloral species distribution, and subsequently, with a nutrient sparing to the animal (Tindall et al., 1985). In other words, the animal puts fewer resources into supporting an indigenous microfloral population and more into growing its own tissues. However, the role of sulfonamides as growth promoters has been attributed to the sparing of energy by fighting less infection, and not to effects in reducing or changing natural microflora (Jukes, 1984). Despite there being a significant cost advantage to using sulfonamides and antibiotics in animal husbandry compared to no antibacterial drug additives (Hayes et al., 2002), there is a growing trend among farmers to reduce or eliminate these compounds in feed due to consumer concerns with food residues. In fact, Sweden has banned the use of antibiotics, growth promoters, and sulfonamides in animal feed, and although the costs in production and animal sickness were initially high, necessary changes in farm management have partly compensated (Kamphues, 1998).

1.3.5. Sulfonamide Residues in Food

Animals treated with a drug will carry its residues in various forms indefinitely. Absolute terms like "no residue level" cannot be used scientifically to describe drug residues in treated animals, because the residue may persist, albeit at infinitesimal and undetectable concentrations. Even at low concentrations, adverse reactions to drugs like

sulfonamides – as either acute hypersensitivity or chronic sub-clinical responses - cannot be overlooked. Hypersensitivity has been associated with penicillin residues in ingested milk and meat (Borrie and Barrett, 1961b; Guillet et al., 2003; Moneret-Vautrin, 2003; Neugut et al., 2001; Schwartz and Sher, 1984; Siegel, 1959a; Wicher et al., 1969). There is no conclusive evidence that sulfonamides have caused any hypersensitivity in people due to consumption of their residues in food (Huber, 1986). However, due to sulfonamides' chemical persistence and also to the hydroxylamine metabolite's tendency to bind protein and become immunogenic in therapeutic situations (Cribb et al., 1990), it is theoretically possible that a sulfonamide-hypersensitized individual could have an adverse reaction to the ingestion of low-dose sulfonamides from food (Bevill, 1984; Burgat-Sacaze et al., 1986; Huber, 1986; Rehm et al., 1986). Circumstantial evidence indicates immunological mechanisms are important in the pathogenesis of inflammatory bowel diseases (Cuvelier et al., 1994). Since these disease processes can be asymptomatic - making the sub-clinical cause difficult to link to the effect - it is important for researchers to investigate compounds such as sulfonamide food residues, as long as they are used therapeutically in human medicine, agriculture and aquaculture.

When widespread sulfamethazine prevalence in the American milk supply was documented in the late 1980s, there was concern due to its structural similarity to dapsone (4,4'-diaminodiphenylsulfone), a known carcinogen (Charm et al., 1988; Griciute and Tomatis, 1980). In 1958, the "Delany amendment" of the United States Food, Drug, and Cosmetic Act, prohibited the use of any food additive that was found to induce cancer in people or animals. As the testing sensitivity of carcinogens improved, many potentially useful substances, like sulfamethazine, were threatened to be banned. Although high doses of sulfamethazine caused cancer-like effects in rat thyroid (Littlefield et al., 1990), it was ultimately shown not to be a human carcinogen, and was allowed for continued use in agriculture (Poirier et al., 1999a). Although formerly used in beekeeping, sulfathiazole is no longer an accepted means to prevent and treat American Foulbrood (*Paenibacillus larvae*) in most countries due to its ability to show up and persist in honey of treated bee colonies (Belliardo, 1981). The current analytical limits of detection using available confirmatory techniques determine if a honey containing sulfathiazole is rejected or not.

Despite the existence of internationally recognized scientific processes (based on statistical risk assessment of toxicological data) to determine allowable concentration and daily intake limits for residues in food (FAO of the United Nations, 2001), no references were made to specific toxicological investigations in establishing the safe limits for sulfamethazine (Rehm et al., 1986) (100 ng/L is the maximum residue limit (MRL) for sulfamethazine in meat, and 10 ng/L is MRL later established for milk). In retrospect, the laws made in Canada and the United States may have been established arbitrarily based on the technological advances of the time (Zomer et al., 1992).

During the 1970s and the early 1980s the percentage of slaughtered pigs, which contained sulfamethazine residues exceeding the United States FDA tolerance of 100 ng/L in meat tissues, was between 10% and 15%. The identifying factors were investigated and reported as follows: shipping pigs for slaughter before the prescribed 15 day waiting period, improper feed mill mixing practices or delivery errors, and usage of medicated feeds on the wrong domestic animal accounted for 94% of the violations (Bevill, 1984). Residue levels exceeding regulations were also found in kidney and liver of pigs that were exposed to excreted sulfonamides from treated pigs. The United States pork industry addressed these problems and reduced the violations to less than 0.4% by the late 1980s (Meeker, 1989). Although sulfonamide use in agriculture and residue prevalence in animal food products have been dramatically reduced, monitoring of animal products continues, especially for products like milk and honey, where illegal use can contaminate whole product batches pooled for processing and packaging (Larocque et al., 1990).

Most recently, concerns with sub-therapeutic treatment of antimicrobials have focused on the emergence of drug resistant bacteria to human and animal pathogens (Berends et al., 2001; Franco et al., 1990; Kunin, 1993; Mazel and Davies, 1999), with food being a possible vector to transmit resistant bacteria to humans from animals (Witte, 1998). Although research has linked the use of antibiotics in agriculture to the emergence of antimicrobial-resistant food borne pathogens, debate still exists as to whether the potential to transfer drug-resistance to human microflora poses a significant risk (Phillips et al., 2004). The important question is, "do resistant populations of bacteria represent independent or common genetic pools?" The selection of resistant bacteria in food

animals could have potential human health implications in the following ways: (1) by human consumption of contaminated drug-resistant bacteria on food and the consequent transfer of resistance determinants to commensal and pathogenic bacteria in the gut; (2) by transference of drug-resistant bacteria on food to a human causing an infection that requires antimicrobial treatment and therapy is compromised; (3) by antibiotics that remain as residues in food product, which allows for the selection of drug-resistant bacteria after the food is consumed (McDermott et al., 2002). Sulfamethazine resistance has been demonstrated by fecal bacteria in swine receiving medicated feed (Aalbaek et al., 1991; Brun et al., 2002; Maynard et al., 2003; Perreten and Boerlin, 2003; Sorum and L'Abee-Lund, 2002; Welch and Forsberg, 1979). Transferable multi-drug resistance elements – conferring tetracycline, sulfonamide, and streptomycin resistance – have been demonstrated in normal flora from pigs similar to those commonly found in clinical isolates of human origin (Sunde and Sorum, 2001). This finding is evidence for the exchange of resistance factors between human pathogens and normal flora of agricultural animals.

1.3.6. Sulfonamide Residues in the Environment

Sulfonamides detected in the environment

Environmental residue analysis of pharmaceutical compounds is a relatively new research focus at universities and regulatory agencies (Daughton and Ternes, 1999; Lange and Dietrich, 2002; Stan and Heberer, 1997). Recently, sulfonamides have been identified in waterways (Battaglin et al., 2000; Furlong et al., 2000; Hartig et al., 1999; Heberer et al., 2002; Heberer, 2002a; Heberer, 2002b; Hirsch et al., 1999; Kolpin et al., 2002; Lindsey et al., 2001; Yang and Carlson, 2003) and other environments (Haller et al., 2002; Pfeifer et al., 2002; Thiele, 2000) associated with human activity (Figure 1.6 shows possible routes for pharmaceuticals to enter the environment).

The largest proportion of sulfonamides entering an ecosystem is the N^4-acetyl metabolite, which have neither bacteriostatic activity nor capability of covalently binding proteins to become immunogenic. Unlike the chemically inert sulfonamide bond, the acetyl moiety of sulfonamides is readily hydrolyzed by acid or base as is done routinely

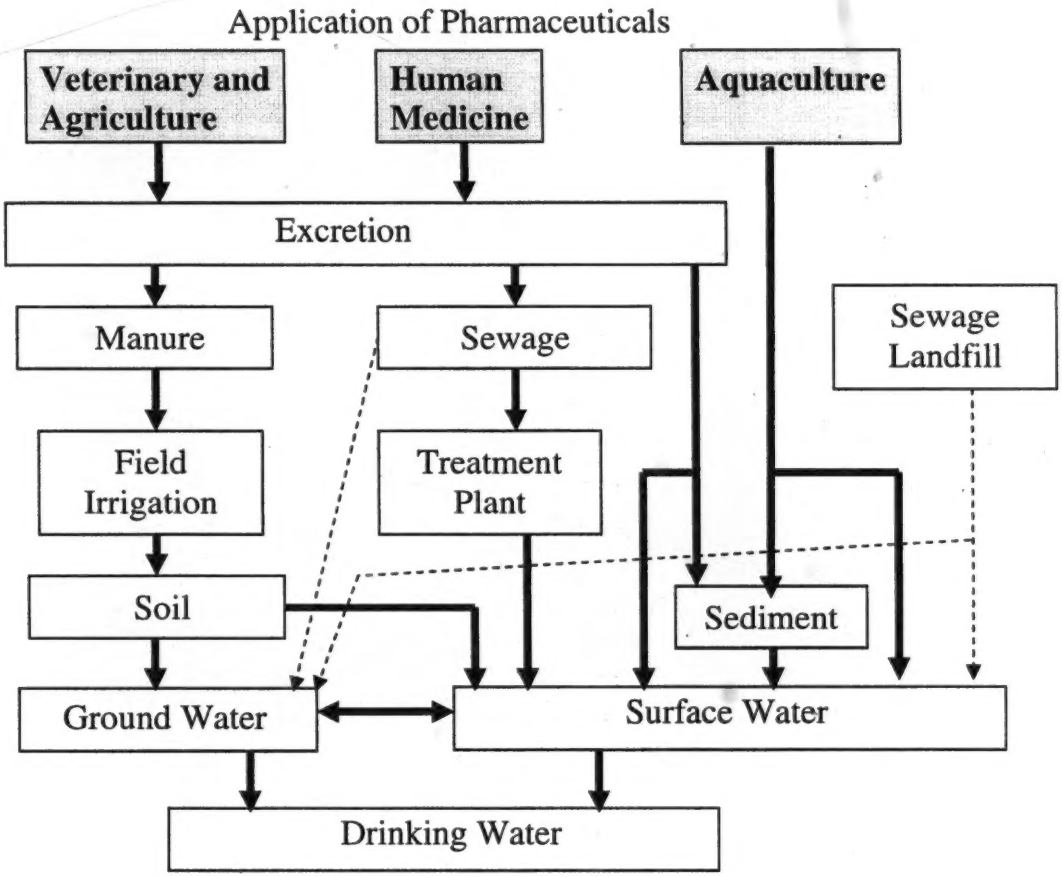


Figure 1.6. Possible pathways of pharmaceuticals entering water systems. Dotted arrows indicate unintentional leakage of sewage or landfill systems. (adapted from Hirsch et al., 1999).

for their colorimetric quantification. In animal tissues, the N^4-acetyl metabolite is in equilibrium with the parent sulfonamide by enzymatic hydrolysis, however, it is not known if the same enzyme systems are used for both acetylation and deacetylation (Vree and Hekster, 1985). These hydrolytic enzyme systems also exist in environmental microbes, since the N^4-acetyl metabolite was shown to convert to the parent compound when placed in manure (Berger and Brünung-Pfaue, 1986), and the concentration of this same metabolite was found to be much lower than the parent sulfonamide in farm animal manure samples (Haller et al., 2002). It is important to be aware of this conversion process, since regeneration of the parent sulfonamide affects the total bacteriostatic activity in the waste lagoon environment at the farm, with associated implications to be discussed later in this section.

Sulfonamide environmental degradation

Metabolic degradation of sulfonamides refers to the actual cleavage of the sulfonamide bond itself, as opposed to phase I and II hydroxylation/conjugation metabolic reactions responsible for facilitating sulfonamide elimination from the body. The only evidence for sulfonamide biodegradation, was demonstrated indirectly in a laboratory activated sludge experiment reporting the ability to systematically "condition" or select bacteria to "degrade" sulfonamides (Ingerslev and Halling-Sorensen, 2000). However, this experiment relied upon decreasing drug concentrations as a measure of degradation, with no direct evidence of sulfonamide breakdown products. It is very likely that sulfonamides were not degraded, but rather were somehow conjugated or changed by the microbial environment making them less recoverable by the initial solid phase extraction system used. The chemical synthesis of the sulfonamide bond in these antibacterial compounds is extremely stable (Pawelczyk and Zajac, 1976), and unlike antibiotics, are not made with biological enzyme systems. It seems unlikely biological systems would evolve mechanisms to biodegrade this stable conformation, and thus sulfonamides in the environment have the capability to be persistent.

Sulfonamide resistance bacterial reservoirs and vectors on the farm

The development of drug resistant bacteria in food animals is a concern from both food and environmental contamination perspectives. In animal husbandry, sulfonamides are routinely given to animals at a rate of 110 µg/L in their feed (Canadian Food

Inspection Agency, 2003). It is reasonable that their concentration in environments immediately surrounding the farm animals and waste lagoons holding farm animal excrement - where a ten to one hundred fold dilution of the feed occurs (see chapter 5 introduction) - could be in the low µg/L range. *In vitro*, sulfonamides still exert their bacteriostatic effect in the low µg/L concentration range based on the minimum inhibitory concentrations documented for several sulfonamides against various enteric microorganisms (Mengelers et al., 1989a; Mengelers et al., 1997). Therefore, sulfonamides in the immediate farm environment are capable of continuing to exert bacteriostatic effects and consequently, in selecting or promoting sulfonamide-resistant strains of bacteria. Also, a bacterium can be resistant to several unrelated antimicrobial agents, and the selection of one particular resistance trait can select for all (George, 1996; Robert, 1996). Theoretically then, conditions that are conducive to selecting and harbouring sulfonamide-resistant bacteria, may concomitantly select for resistance to other antibiotics of much greater relevance to human and veterinary medicine. Agricultural bacterial gene reservoirs become potential vectors of drug-resistance (Berends et al., 2001). Manure containing antibacterial compounds spread onto agricultural land also has the potential to affect the soil's microbial ecosystem, and possibly transfer drug-resistance to the soil bacterial community (Seveno et al., 2002).

Sulfonamide-contamination of crops grown in manure-fertilized fields

Concerns also arise with the spreading of sulfonamide-containing manure onto agricultural fields used for food production. Miglore et al. (1995, 1996a, 1996b, and 1998) demonstrated that sulfadimethoxine could inhibit development of barley and other plants grown in soil, and the sulfonamide was shown to incorporate into the root, stem and leaves of the plants. The soil concentration of sulfadimethoxine was found to be approximately 100 µg/L, or about 10 to 100 fold higher than normally anticipated if manure from treated animals were spread on a field, based on assumptions and findings discussed in chapter 5 of this thesis. The sulfonamide levels in the roots and leaves/stalks were 79 µg/L and 18 µg/L, respectively, well above the 100 ng/L MRL set for pig tissues. Regardless of the abnormally high sulfonamide concentration used in this experiment, it clearly demonstrated that plants can incorporate sulfonamides from the soil and established the potential for sulfonamide bioaccumulation. Depending on the local

geography and soil structure, the recent weather conditions, the agronomic practices and the rates of manure application, one can imagine possible scenarios that would concentrate sulfonamide applied around a growing plant. Further research could demonstrate if plants grown under normal agronomic practices with pig farm manure under a variety of soil, geographical and climatic conditions could bioaccumulate sulfonamides to levels that are higher than MRLs set for food.

Movement of sulfonamides into waterways

Medications given to people and animals are excreted in their urine, enter into waste treatment systems, and enter the waterways in these effluents (Drewes et al., 2002; Hirsch et al., 1999; Khan and Ongerth, 2002; Metcalfe et al., 2003; Soulet et al., 2002). Ultimately, the more polar and persistent pharmacologically active compounds have been detected in drinking water from these processes (Heberer et al., 2002; Jones et al., 2001; Stan and Heberer, 1997). Manure from farms that give animals drug-treated feeds, are spread onto agricultural fields as fertilizer, and they leach out through the soil and eventually reach rivers by surface or underground water drainage. Although pharmaceuticals have not been documented in Canadian drinking water, agricultural pesticides have (Ritter et al., 2002), and the same principles are involved in transporting both types of organic pollutants. Many factors are important to consider in determining if a drug could pose an environmental risk when applied to the soil (Poiger et al., 2003; Tolls, 2001). Sulfonamides are considered highly mobile in soils due to low adsorption to clay loam and sandy loam soils (Boxall et al., 2002). Their mobility through soils may be pH dependent, with large solubility changes noted in solutions with acidity near the pK_a of a sulfonamide's N^1 proton (Vree and Hekster, 1987). In general, chemicals with a greater hydrophobic nature and less water solubility adsorb more to soils, as can be described by the sorption coefficient (K_d) in Equation 1.1.

Equation 1.1. K_d solid = C_s/C_{aq}; where C_s and C_{aq} are the concentrations of a compound in the sorbent and aqueous phases, respectively.

Organic compounds with more hydrophobic character tend to have high K_d and bind more to soil than be solubilized in water. Compared to their protonated forms, salts

of organic compounds tend have a lower K_d in soil, because their charged forms adhere less to the hydrophobic soil. Depending on the N^1 substituted group and the pH of the solution, sulfonamides generally have a K_d close to unity, whereas tetracyclines have K_d values around 1000 (Tolls, 2001), meaning that a tetracycline binds to soil 1000 fold more than a sulfonamide. Although tetracyclines are used to a much greater scale in human and veterinary applications (Thiele-Bruhn, 2003), they were detected about ten times less frequently than sulfonamides in a transcontinental American survey that sampled waterways suspected of having contamination (Kolpin et al., 2002). This may be explained in part by tetracycline's higher soil sorption coefficients compared to sulfonamide, but also by its ability to biodegrade (Aga et al., 2003; Peterson et al., 1993). In another American survey comparing 144 surface and groundwater samples, tetracyclines were found only in surface water samples, whereas sulfonamides were found in both surface water and groundwater samples, indicating sulfonamides can readily move through soils in the water environment, whereas tetracyclines do not (Lindsey et al., 2001). The more hydrophobic drugs, like the tetracyclines, may accumulate in soils with repeated manure applications (Hamscher et al., 2002). The possible microbiological implications of concentrating antibiotics adsorbed to soil surfaces, where large numbers of bacteria exist, are uncertain.

Another interesting aspect of the Koplin et al. (2002) survey sampling 104 waterways, was that sulfamethoxazole predominated the survey samples (12.5% prevalence with an average concentration of 0.15 ng/L) compared to sulfamethazine (4.8% prevalence with an average concentration of 0.02 ng/L). Sulfamethoxazole is a long-acting drug used commonly in human medicine to treat urinary tract infections, whereas sulfamethazine is associated with veterinary medicine and feed additives in animal husbandry. Although the survey did not correlate the source of the water to the type or quantity of residues found, this type of information may be important in determining the relative contribution of pharmaceutical pollutants by agriculture and urban centers.

1.4. METHODS OF ANALYSIS OF SULFONAMIDE ANTIBACTERIAL COMPOUNDS

The enormous advancements in computer technology since the 1980s are largely responsible for recent improvements in control and automation of residue analysis methodologies. Increased sensitivity and specificity of techniques have allowed for miniaturization and reduced the extensiveness of sample clean-up. Before the 1980s, samples for routine residue analysis were typically 50 g and were extracted with large volumes of organic solvents. Separatory funnels, volumetric and evaporation flasks, and glass pipets were used together to manually extract, evaporate, separate, and transfer sample fractions, and analytical systems had to be attended continually by the operator. Although these procedures are still in use today for method development or in non-routine analysis, contemporary routine residue analysis is typically automated and involves parallel batch extractions of samples of less than 1 mL or 1 g in vials or test tubes. Parallel batches of samples can be processed in centrifugal evaporators and solid phase extraction (SPE) systems prior to analyte isolation and identification. Advances in automated derivatization, dilutions/additions, on-line column-switching technologies, and computer-integrated instrumentation control and monitoring, have revolutionized the capability and potential of residue analysis (O'Keeffe, 2000).

1.4.1. Non-Confirmatory Methods

There are a variety of methods available for sulfonamide residue analysis, and although most literature has focused on food, the recent trend has been on environmental samples. Table 1.1 summarizes techniques for food or environmental sample sulfonamide residue analysis reported in the literature, together with the analytical principles and specific advantages and disadvantages of each. The Bratton-Marshall assay, described 75 years ago (Bratton et al., 1939), still has utility today as either a quick and simple means to quantify sulfonamides at the research laboratory, or as a sensitive visualization technique in combination with other methods. Many methods, like thin layer chromatography, immunological and microbiological assays, are noted for their great efficiency and low cost per sample. The enzyme-linked immunosorbent assay (ELISA) in particular is remarkably sensitive especially considering it requires very small

Table 1.1. Evaluation of analytical methods for sulfonamide residues in food or environment

Method Name	Principle of Method	Advantages (+) and Disadvantages (-)	Literature References for Residue Analysis
Bratton-Marshall Assay (BM Assay)	Colorimetric determination of a diazo sulfonamide derivative at a wavelength of 545 nm. Diazo derivatization of free aromatic amino with nitrite in acid, followed by reacting with N-(1-napthyl)ethylenediamine dihydrochloride to give a coloured diazo compound	(+) Fast, simple, and inexpensive (-) Low sensitivity (low µg/L limit of detection). Very non-specific and capable of reaction with most aromatic amine compounds	(Bratton et al., 1939; Low et al., 1989; Marshall Jr. and Babbitt, 1938; Mount et al., 1996; Whelpton et al., 1981)
Thin Layer Chromatography (TLC)	Separation of compounds by differential mobility in solvent phase through a thin solid phase (usually silica gel) applied to a glass plate. Ultra violet visualization of sulfonamides or derivatization of sulfonamide with Bratton Marshall reaction. Thin layer radio-chromatography can be used for metabolism and breakdown studies.	(+) Fast, simple, and inexpensive (-) Sensitivity and specificity is dependent on non-specific visualization methods	(Agarwal, 1986; Giovanardi et al., 1994; Horwitz, 1981a; Sherma, 2003; Thomas et al., 1983; Unruh et al., 1993)
Capillary Zone Electrophoresis (CZE)	Separation of charged compounds based on their relative eletrophoretic mobilities for a given buffer system in a capillary tube, and detected by UV absorption.	(+) Sensitive (low ng/L limit of detection. (-) Complex equipment necessary, and speed of system is limited by serial electrophoretic run-time.	(Ackermans et al., 1992; Fuh and Chu, 2003; Ng et al., 1993)

Table 1.1 cont'd... Evaluation of analytical methods for sulfonamide residues in food or environment

Method Name	Principle of Method	Advantages (+) and Disadvantages (-)	Literature References for Residue Analysis
Enzyme-linked Immunosorbent Assay (ELISA)	ELISA is used in a competitive format, where anti-sulfonamide antibodies bind to sulfonamide competitor from a sample, and this inhibits antibody binding to a solid-phase immobilized reference sulfonamide. Antibody binding to the reference sulfonamide is colorimetrically measured directly or indirectly using enzyme-conjugate labels, and is a function of sulfonamide concentration in the sample.	(+) Very sensitive (ng/L to sub ng/L limits of detection). Small, unconcentrated sample volumes required, and very efficient and conducive to high through-put screening. Little if any pre-analysis preparation required. Kit forms available for field testing. (-) Cross-reactivity is often encountered amongst related sulfonamides and metabolites, and makes this not specific enough for positive identification. Also, sample matrix effects cause false positives. Large replicate variation.	(Assil et al., 1992b; Braham et al., 2001; Haasnoot et al., 2000b; Ko et al., 2000; Lee et al., 2001; Muldoon et al., 1999; Muldoon et al., 2000; Sheth and Sporns, 1991; Thomson and Sporns, 1995)
Immunobiosensor	Binding and dissociation of sulfonamide to an immobilized antibody are monitored by shifts in light signal transduction. Surface Plasmon Resonance (SPR) device is reported for sulfonamides. These optical biosensors use an evanescent filed to measure changes or refraction index on the sensor surface.	(+) "Real time" characterization of biomolecular interactions without labelling reactants. Sensitive (low ng/L) and high throughput capability (-) As with ELISA, all the cross-reactivity and false positive problems associated with antibodies	(Bjurling et al., 2000; Crooks et al., 1998; Situ et al., 2002)

Table 1.1 cont'd... **Evaluation of analytical methods for sulfonamide residues in food or environment**

Method Name	Principle of Method	Advantages (+) and Disadvantages (-)	Literature References for Residue Analysis
Microbiological Inhibition Assays	Inhibition of *Bacillus* genus bacterial growth.	(+) Sensitive (ng/L limits of detection) and multiple sulfonamide detection capability (-) Not antibacterial-drug specific, nor can it detect inactivated sulfonamide metabolites. Slow, requiring time for bacterial growth.	(Bogaerts et al., 1981; Charm et al., 1988; Read et al., 1971; Vermunt et al., 1993)
Charm II Microbiological Receptor Assay	Similar in principle to the ELISA, except using a microbial receptor instead of an antibody. Sulfonamide from a sample inhibits a labelled sulfonamide tracer from binding microbial receptors, and the degree of this inhibition is a function of a sample's sulfonamide concentration.	(+) Sensitive (ng/L limits of detection) and capable of detecting multiple sulfonamides. Fast, convenient, and easy to use. (-) Cannot differentiate sulfonamides	Charm Sciences, Inc., Lawrence, MA

Table 1.1 cont'd... **Evaluation of analytical methods for sulfonamide residues in food or environment**

Method Name	Principle of Method	Advantages (+) and Disadvantages (-)	Literature References for Residue Analysis
Gas Chromatography with Mass Spectrometric Detection (GC-MS)	Following pre-analysis purification, concentration, and derivatization, samples are separated by their differential solubility in the gas phase of a capillary column. Eluted compounds are further separated and fragmented for identification in the mass spectrometric analyzer(s).	(+) Sensitive (low ng/L limits of detection) and accurate, especially with tandem mass spectrometry technology (-) Expensive, time consuming, and requires highly trained operator. Pre-analysis concentration and purification required. Usually requires an extra derivatization step. Rate limiting, "bottle neck" step of serial chromatographic run-time (10-30 min).	(Cannavan et al., 1996; Ito, 2003; Mooser and Koch, 1993; Reeves, 1999; Tarbin et al., 1999)
High Performance Liquid Chromatography with Mass Spectrometric Detection (HPLC-MS)	Following pre-analysis purification, and concentration, samples are applied to a column in a solvent system that allows them to be retained by the solid phase packing, which can have a variety of surface chemical properties. The compounds are then eluted and separated based on their differential solubility in a mobile liquid phase. Eluted compounds are further separated and fragmented for identification in mass spectrometric analyzer(s).	(+) Sensitive (parts per trillion to low ng/L limits of detection) and accurate, especially with tandem mass spectrometry technology (-) Expensive, time consuming, and requires highly trained operator. Pre-analysis concentration and purification required. Rate limiting, "bottle neck" step of serial chromatographic run-time (10-30 min).	(Abian et al., 1993; Cavaliere et al., 2003b; Doerge et al., 1993; Haller et al., 2002; Hartig et al., 1999; Hirsch et al., 1999; Ito, 2003; Lindsey et al., 2001; Pfeifer et al., 2002; Porter, 1994; Sherma, 2003; Verzegnassi et al., 2002)

sample size (less than 1 mL) with almost no pre-analysis preparation, and is capable of high throughput analysis of hundreds to thousands of samples per day. Although some sample matrix interference problems are encountered in sulfonamide ELISA analysis, this can usually overcome by a simple pre-analysis dilution or extraction steps (Crabbe et al., 1999). Yet these assays have problems to varying extents with specificity, and cannot be used alone to confirm the identity of a compound. These are excellent screening tools for incurred food or environmental residues, but will not be discussed in depth here, where the focus is on technologies capable of positively identifying an analyte.

1.4.2. Gas Chromatography and High Performance Liquid Chromatography

Gas chromatography (GC) and high performance liquid chromatography (HPLC) techniques are capable of resolving complex analyte mixtures, and are used widely to detect and quantify sulfonamides or other organic residues. Ultraviolet light absorbance (at wavelengths of 254 and 270 nm) is often used to detect sulfonamides in GC or HPLC. However, confirmatory residue analysis usually requires the combination of one of these chromatographic techniques and then detection by mass spectrometry (MS). These coupled or hyphenated analysis methods, like GC-MS or HPLC-MS, tend to be very expensive, time-consuming, and require highly trained individuals to maintain and operate the equipment. They require pre-analysis concentration and purification by liquid/liquid extraction and/or solid phase extraction, which will be discussed in more detail in the next section. Most mass analyzers are not tolerant of sample impurities, so the initial on-line chromatographic step, such as GC or HPLC, serves to separate the analytes from each other and from impurities, prior to further characterization by MS. This chromatographic step is the fundamental "bottle-neck" of the analysis process, and ultimately limits the production capability of the most sophisticated of these automated systems to about 100-200 samples per day in automated units running 24 h/day (Bartolucci et al., 2000; Koeber et al., 2001).

GC is not very suitable for the analysis of polar or ionic compounds, but if the N^4 and/or N^1 nitrogens are methylated, sulfonamides can be separated and analyzed well by GC-MS (Matusik et al., 1990; Mooser and Koch, 1993; Reeves, 1999; Tarbin et al., 1999). There is a great variety of stationary phases and columns available, and together

with infinitely variable temperature programs and specific mass analyzer systems, GC-MS is capable of detecting and quantifying sulfonamides residues in the sub-ng/L range.

HPLC-MS has become a common method for identifying sulfonamides in food and environmental samples (Doerge et al., 1993; Giovanardi et al., 1994; Hartig et al., 1999; Heberer et al., 1998; Hirsch et al., 1999; Kishida and Furusawa, 2001; Kolpin et al., 2002; Pfeifer et al., 2002; Porter, 1994; Soulet et al., 2002; Zwiener and Frimmel, 2004a; Zwiener and Frimmel, 2004b). The technical problems associated with interfacing HPLC with mass spectrometers, such as the high flow rates of HPLC, the salts associated with their buffering systems, and the involatility of analytes have only recently been overcome (Niessen and Tinke, 1995). HPLC is a versatile chromatographic process that can use many different solid phases to concentrate and purify more hydrophilic analytes. In such cases, reversed phase HPLC is normally used where the stationary liquid phase is hydrophobic with a more polar mobile phase. Other systems based on adsorption, size-exclusion, ion-exchange and immunoaffinity solid phases are also used (Porter and Patel, 2000). The analytes are concentrated on the column and then selectively eluted on-line into the MS system.

1.4.3. Mass Spectrometry Coupled Systems

Mass spectrometers have the advantage over other detection systems in that they give both structural and quantitative information about an analyte. Functionally, they can be considered in three parts: (1) the ion source, which volatilizes the analyte and gives it a charge; (2) the analyzer, which separates the analytes by mass; (3) and the detector. The main ionization techniques used for GC-MS are electron impact and chemical ionization. HPLC-MS uses atmospheric pressure ionization, such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI). Mass analyzers separate analytes based on their mass to charge ratio (*m/z*). The three most common instrument types used are: (1) magnetic-sector instruments that use a magnetic field to separate ions as a function of their momentum; (2) transmission quadrupole, which uses a quadrupole field to allow ions of a given *m/z* to pass from the ion source to the detector while destroying all others; (3) and the quadrupole ion-trap, which uses a quadrupole field to store ions and then destabilize them one *m/z* value at a time, and thus obtain a mass spectrum. The time-of-flight instruments, which separate ions based on the time they take to travel a

flight tube, were the first type of analyzer used in mass spectrometry. Although they are not currently used in residue analysis of complex sample matrices, they will be discussed in detail later in section 1.5.2, in the context of this thesis.

Often chromatographic separation is coupled to tandem mass spectrometers (e.g. HPLC-MS-MS) to give more definitive structural information. The first mass spectrometer usually uses a transmission quadrupole that filters out an ion of a specific *m/z* value. The isolated ion(s) then passes through a collision chamber, causing further fragmentation, the products of which are analyzed by a second mass spectrometer. Analyte separation by chromatography, and then by a primary mass filter, and the subsequent mass identification of each fragment by mass confers a high degree of confidence in analyte identification (March, 1997; Porter and Patel, 2000).

1.4.4. Methods of Pre-analysis Extraction, Concentration and Purification

When they occur in foods or environmental samples, organic residues usually exist in trace quantities at the ng/L level, and lower. These low concentrations make residue analysis inherently difficult. The objective of pre-analysis treatment is to eliminate or decrease sample matrix effects that can interfere with subsequent analysis, and/or to concentrate the analytes to achieve the required limit of detection.

Sampling and Storage

Collected samples are usually divided into two or three portions, ideally after homogenization. Special attention is required to prevent the contamination of uncontaminated or less contaminated samples with highly contaminated samples or standard references, either at the field or in the laboratory. Proper sample packaging, identification, and cleaning of field and laboratory equipment between sampling is essential to avoid contamination. Samples are usually stored at -20°C to minimize the destruction of the residue to endogenous enzymes and chemical processes in the sample. The sample is thawed immediately prior to analysis.

Primary Extraction

Liquid-liquid extraction is the classical method for sample extraction and clean-up. For an aqueous sample, an immiscible organic solvent is added and manually or mechanically shaken. For a solid sample, it is usually homogenized in an aqueous buffer

system, and then this suspension is manually or mechanically shaken with an immiscible organic solvent. The aqueous and organic solvents are then separated to acquire the upper analyte-containing organic layer. Liquid-liquid extraction is traditionally laborious, requires large solvent volumes and is prone to cross contamination, whereas modern systems allow for batch processing of samples with disposable containers and solvent evaporation systems, thus decreasing contamination errors. Samples containing lipid that can interfere with subsequent solid phase extraction, are commonly defatted with hexane, before extraction with diethyl ether, methanol, ethanol, or acetonitrile (and other various solvents) to recover sulfamethazine and acetylsulfamethazine residues in the 80% range (Guggisberg et al., 1992; Haller et al., 2002; Pfeifer et al., 2002).

Solid Phase Extraction

Purifying water by percolating it through layers of sand, loam, and charcoal, is an ancient example of solid phase extraction (SPE) technology used to separate compounds from a liquid phase. SPE has become very sophisticated, where solid phases, their containment and handling systems, and the accompanying liquid systems for washing and eluting, are all highly controlled and specific in their design. Solid phase extraction systems relevant to drug analysis include the cartridge or microplate forms, and matrix solid phase dispersion (MSPD). MSPD involves adding the sorbent material to a solid or viscous sample, which acts both as an abrasive disruption agent and as an extracting adsorbent. Instead of extracting a solid sample with liquid first before SPE, MSPD combines these steps, and then the dispersed sample/sorbent combination can be placed in a column for washing and elution (Barker, 2000). This technique has been used for sulfonamide residue recovery from meat and milk (Kishida and Furusawa, 2001; Tamura et al., 1994; Vanpoucke et al., 1991)

Cartridges or microplates containing bonded phase partition adsorbents are the most common form used in organic residue analysis. The surface chemistry of the solid phase is derivatized or altered to perform a specific extraction function. The most common example of SPE in organic compound residue analysis is octadecyl (C_{18}) on silica. One the greatest drawbacks of the C_{18} material is that it requires conditioning with a water-miscible solvent like methanol, the conditioning is depleted with larger aqueous samples, and the exchange material becomes dysfunctional if allowed to run dry. Ion exchange systems such as the basic aminopropyl groups on silica can take advantage of

the anionic nature of sulfonamides in their purification (Pfeifer et al., 2002). Newer polymeric reversed phase sorbents are now available that possess both hydrophilic and hydrophobic functional groups, and remain wetted with water, and are commonly used for sulfonamide extraction (Kolpin et al., 2002; Lindsey et al., 2001). The advantage with these hydrophobic-lipophilic balance systems (HLB Plus™, Waters, Milford, MA) is that they do not require conditioning, and can be tailored to combine ionic exchange, hydrogen bonding, and hydrophobic interactions to optimize selective sulfonamide recovery.

SPE of sulfonamides requires awareness of their chemistry. They have a tendency to exist reversibly bound to proteins, so preliminary measures, like protein precipitation or solvent extraction, may be necessary to liberate sulfonamide before SPE. Also, sample pH is an important consideration when using hydrophobic or ionic mechanisms in SPE. For instance, the water solubility of SMT decreases sixteen fold from pH 7.0 to pH 5.5 (close to its isoelectric point at pH 5.0), and, relative to its N^4-acetyl metabolite (NA-SMT), has a 5 fold greater solubility at pH 7, yet relatively only one half the solubility of NA-SMT at pH 5.5 (Vree and Hekster, 1987). In this case, selection of an extraction buffer around the isoelectric point of SMT would greatly facilitate its recovery by reverse phase SPE. Alternatively, an extraction buffer above the pK_a of the N^1 nitrogen would facilitate its extraction using an anionic exchange resin.

Immunoaffinity and Molecular Imprinted Polymer Chromatography

Aside from their usefulness in residue analysis using ELISA, antibodies have been routinely used for the enrichment of residues prior to analysis by immunoaffinity chromatography. Immunoaffinity columns (IACs) are made of analyte-specific antibodies covalently attached to a solid phase, and they are used to specifically remove analytes from interfering sample matrix effects prior to analysis. The literature is replete with examples in organic pollutant and drug residue analysis in food and environmental samples, where IACs are commonly coupled on-line to GC or HPLC (Delaunay et al., 2000; Hennion and Pichon, 2003; Pichon et al., 1997; Stevenson, 2000). The high affinity and specificity of the antibody-antigen interaction, enables an IAC to selectively concentrate and/or extract a compound or a class of compounds in a simple single step. Before the current application of IAC for sulfonamide extraction from environmental samples (chapters 4 and 5), IACs have been reported for purification of sulfonamides

from biological samples such as milk, urine, and meat (Crabbe et al., 1999; Heering et al., 1998; Li et al., 2000; Martlbauer et al., 1996). Further discussion about IAC theory is presented in a separate section devoted to its consideration (section 1.5).

An alternative to the expensive development of IACs is the use of molecularly imprinted polymers (MIP), or "synthetic antibodies" as they are known. Molecular imprinting involves heavily cross-linking a resin in the presence of an analyte template compound. The template is then thoroughly washed, and the resultant cavities are complementary to the size and shape of the analyte. The MIP and analyte interact through hydrogen bonding, hydrophobic and ionic interactions, and van der Waals forces, in much the same way as an antibody does with an antigen (Hennion and Pichon, 2003). Binding constants for MIP systems have been reported as high as 10^9 L/mol, comparable to those observed for antibody-antigen interactions (Andersson et al., 1995). Many problems are being addressed with this new technology, and this area shows much promise for MIP affinity chromatography, with advantages over IAC being their minimal cost and time of preparation, and a high thermal and chemical stability. A MIP system has been developed for sulfonamides, and is being explored with the intention of using it in MIP SPE (Zheng et al., 2002).

1.4.5 Regulatory Aspects of Residue Analysis

Regulatory methods are those that have met suitable performance criteria as designated by national and international authorities. Methods are usually classified as levels I, II, and III, corresponding to screening, determinative, and confirmatory analyses (Macneil and Kay, 2000). Level I methods are based on techniques like ELISA and microbial receptor kits that are usually rapid and give qualitative or semi-quantitative results. Level II methods based on separation instrumentation like HPLC or GC or capillary electrophoresis, are used primarily to quantify the analyte, but do not usually provide unequivocal identification. Level III methods provide unequivocal analyte identification, and include the hyphenated chromatographic-mass spectrometric techniques like GC- or HPLC-MS (MS/MS). Combinations of techniques based on different principles improve the confirmatory nature of a method, such as combining reverse-phase chromatography with MS, or by using immunoextraction or molecular imprint technology as a means of sample clean-up.

Under guidelines from the International Organisation for Standardization, collaborative studies are usually necessary from accredited laboratories. The method should include performance standards, such as limits of detection and quantification, analytical recovery and precision. Also, other criteria should be described like the method's applicability, types of sample matrices, types and concentration ranges of analytes being measured, and critical control points of the assay. From a regulatory point of view, a valid assay result is dependent on many other criteria other than using a valid or recognized methodology. Other factors that need to be controlled are a properly facilitated laboratory, which is accredited by authorities, which uses calibrated equipment operated by qualified and properly trained analysts, which uses appropriate standards of reference, and which adheres to correct sampling collection, storage, and handling methodologies. Faults made at any one of these points may constitute the weakest link of the process, and result in unacceptable error in the final result.

The quantitative assay performance guidelines for residues in food are described by the Food and Agriculture Organization of the United Nations in Table 1.2 (FAO (1993b) Codex Alimentarius, 1993). The limits of detection (LOD) or limits of quantification (LOQ) are defined for each method, and are usually described in relation to either the analyte's signal to noise ratio (usually 3:1 or 10:1, for LOD and LOQ, respectively) or in relation to the standard error of the mean (SEM) (usually 3 X or 10 X the SEM, for LOD and LOQ, respectively). The LOD and LOQ are required to be less than the maximum residue limit (MRL) set by the regulations. Each regulatory body governing residue testing usually has specific protocols for validation of methods. The validation procedure starts at establishing capable analysis of standard solutions at 0.5, 1, and 2 times the MRL in phase 1, then in the second phase fortified sample matrices are analyzed at the same concentrations as the first phase, and the third phase analyzes blind samples (incurred and fortified) by various laboratories. The assay must achieve minimum performance requirements of recovery, accuracy, and precision at each phase.

There is considerable debate about what constitutes confirmatory identity of a compound, but in general for the mass spectrometry techniques, three mass fragments associated with the analyte are monitored and must be present for each positive sample. Tandem mass spectrometry techniques (MS/MS) are inherently more definitive in residue analysis because they are less likely to generate inconclusive fragmentation patterns. The

Table 1.2. Expected assay performance parameters for quantitative methods used in a regulatory program (FAO (1993b) Codex Alimentarius, 1993)

Concentration (µg/kg)	Coefficient of variability (%)	Required accuracy range (%)	Recovery (%)
≤ 1	35	-50 to +20	Not specified
>1 ≤ 10	30	-40 to +20	60 to 120
10 ≤ 100	20	-30 to +10	70 to 110
>100	15	-20 to +10	80 to 110

first MS serves as a filter, before fragments are generated and analyzed by the second MS, which serves to greatly reduce the possibility of confounding analyte identification by unknown molecular weight isomers.

In regard to sulfonamide testing, the MRLs for sulfamethazine (SMT) are 100 ng/L in meat, and 10 ng/L in milk, so the SMT limit of detection and quantification for legal purposes in food analysis should be significantly lower than 10 ng/L, perhaps in the parts per trillion range. Although there are no MRL values for pharmaceutical compounds in environmental systems, a sub-ng/L detection limit for analytical systems would likely be adequate to monitor sulfonamides in the environment. Monitoring ng/L levels of sulfonamides would enable downstream tracking of the fate of these residues in water and soils in ecosystems where they are introduced by agricultural practices or sewage processing facilities at µg/L or high ng/L levels. Although sulfonamides do not have any demonstrated antimicrobial activity at concentrations lower than µg/L levels, monitoring them in the environment at the ng/L level would permit experiments to determine their probable sources and fates, and as models to monitor the fate of similar compounds. Also, monitoring sulfonamides in the environment at concentrations that are below bacteriostatic levels may be important to assist in determining if there are other biological activities associated with these potentially persistent organic pollutants.

1.5. CONSIDERATIONS OF ANTIBODIES RELEVANT TO IMMUNOAFFINITY CHROMATOGRAPY OF HAPTEN RESIDUES

1.5.1. History of Antibodies

It was only a century ago that Paul Erlich recognized the function of a class of proteins in the mammalian defence system, which he called antibodies. Later, antibodies were used as analytical tools, originally, used in agglutination assays to determine the presence of a cell type or to determine the specific antibody titre of blood to an antigen. Later, when radioisotopically-labelled antibodies were shown to be capable of specifically quantifying low levels of human insulin (Yalow and Berson, 1959), the age of the labelled antibody assay was born, and rapid developments followed in radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblotting and cell staining. Other potential uses for antibodies have opened up, such as chemotherapeutic targeting agents in medicine (Abou-Jawde et al., 2003) and synthetic enzyme systems (Wade and Scanlan, 1997). Now, on the cusp of the genetic revolution, antibody technology is rapidly evolving into the 21st century with new potentials to produce virtually unlimited quantities of antibodies of designed specificity (Yau et al., 2003). The course has been charted by gaining genetic control of antibody probes, from the development of monoclonal antibodies in the 1970s (Kohler and Milstein, 1975), to the ability to transgenically clone whole human chromosomes conferring human antibody production in mice (Tomizuka et al., 1997), and to advances in genetic engineering of human antibody fragments through recombinant technologies such as phage-display (Smith, 1985) and ribosome display techniques (Mattheakis et al., 1994).

1.5.2. Immunization with Haptens

The immune system has an extraordinary capability of creating millions of different possible specificities, which are conferred ultimately by different amino acid sequences at the binding site of the antibody molecule (Harlow and Lane, 1988). Antibodies have been well studied structurally and functionally, as have the genetic mechanisms responsible for their generation. An antigen, or immunogen, is any compound that is capable of eliciting a response from an immune system. Small molecules, or haptens, of less than 3000 Da, do not make good immunogens, because

they are not capable of being presented properly to the immune system. Macromolecular systems are required to elicit an immune response; they are large enough to bridge between immune system cellular receptors, a process necessary in antigen recognition and subsequent antibody production.

The generation of antibodies to haptens is relevant to the research of this thesis because sulfonamide drugs, as haptens, are not immunogenic on their own. Antibodies can be generated to a hapten if an animal is immunized with a larger hapten-macromolecule conjugate. Some of the resulting antibodies will have specificity for these haptens. It is critical to recognize two important ideas when using or evaluating anti-hapten polyclonal antibodies in research. Firstly, the antibodies are not raised against the hapten itself, but rather are made to a molecule comprised of the hapten covalently linked to a macromolecule. Portions of the macromolecule and the linker arm used to join the hapten to the macromolecule may be involved in the antibody binding site, and thus affect the specificity. Problems associated with specificity in antibody binding a hapten and its linker arm are referred to as "linker arm effects", and will be thoroughly discussed in context to experimental results in chapters 2 and 3. Secondly, unlike monoclonal or recombinant antibodies that are structurally identical, when antibodies are derived from an immunized animal, it creates a variety of antibodies (polyclonal) that bind a given hapten, or portion of that hapten, in a variety of ways. Inherently, polyclonal antibodies are less specific than monoclonal antibodies because many structural binding strategies exist simultaneously, and the probability of cross-reaction with related haptens is greater. However, when extracting drugs with immunoaffinity chromatography, heterogeneity and cross-reactivity may be an advantage when related drugs and their metabolites are also being analyzed. Also, polyclonal antibodies are known for their higher affinities than commonly reported for monoclonal or recombinant antibodies (Delaunay et al., 2000; Hennion and Pichon, 2003; Yau et al., 2003).

1.5.3. The Anatomy of the Antibody-Hapten Interaction

The most common form of antibody generated and used in analytical systems is the immunoglobin G molecule (IgG) (Harlow and Lane, 1988). MALDI-TOF MS IgG has a molecular weight of about 150 kDa, and has the shape of the letter "Y". It

is made of four protein chains joined by disulphide linkages, and has two domains - Fab and Fc - that can be isolated by enzymatic hydrolysis. An antibody's binding specificity is determined by the amino acid sequences of six peptide loops that make up the hypervariable region at the end of each Fab domain (Figure 1.7). These hypervariable sequences are responsible for the formation of a cleft or pocket that binds a hapten molecule. As already mentioned, the structural variation at the antibody binding site is immense. An anti-insulin antibody can reach out over as large an area as 750 $Å^2$ covering about 11% of insulin, or in contrast for an anti-hapten antibody, it can be a very small pocket, measuring less than 10 Å across or deep, accommodating portions of a small molecule like morphine (Figure 1.8). The portion of any molecule which fits into the binding site of a given antibody is defined as the "epitope". In solution, a hapten binds to an antibody by a "hand-in-glove" fit where intimate contact between the two molecules is stabilized by non-covalent forces such as ionic and hydrophobic interactions, hydrogen bonding, and van der Waals forces. The binding strength of antibody for an antigen is measured by the affinity constant (K_A = [bound antibody-antigen complex]/[antibody]*[antigen]), which is often about 10^9 M^{-1}, but can range from10^5 to 10^{12} M^{-1}. The affinity of an antibody is affected by the solvent type, the pH and ionic strength of the solution, which are important factors in consideration of optimal binding or elution of haptens in immunoaffinity column systems. Since antibodies recognize relatively small epitopes, they often cross-react with similar epitopes on other molecules. The binding constants for antibody-epitope interactions can be very high, simply because the proportion of the hapten molecule engulfed and stabilized by the antibody binding site is much greater than for macromolecular antigens. This can cause problems in eluting haptens from antibodies in immunoaffinity chromatography without using conditions that damage the antibody (Delaunay et al., 2000).

With respect to polyclonal antibody IAC (using unpurified IgG), sulfonamides have been eluted using methanol (Li et al., 2000), and by lowering the pH (Crabbe et al., 1999). Since sulfonamides are weakly acidic (pK_a of N^1 nitrogen = 7.4 for sulfamethazine) and have negative ionic character at neutral pH, it is likely that the anti-sulfonamide antibodies would use a positive charge ionic-coupling to stabilize the anionic sulfonamide form. Lowering the pH would make the sulfonamide neutrally charged, and thus break the coulombic stabilization by the antibody, allowing it to elute

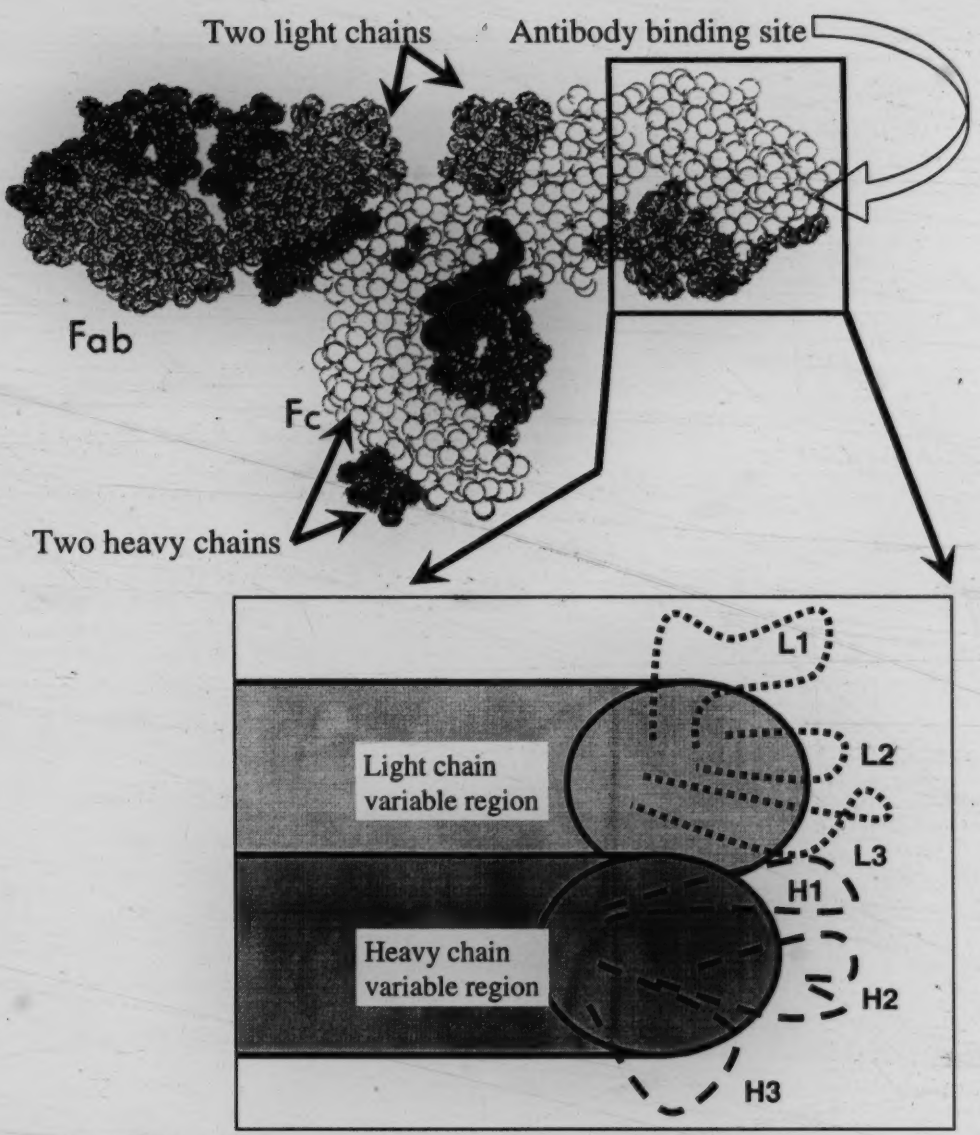


Figure 1.7. A space-filling model of an immunoglobin G molecule (IgG).

Two heavy chains (darkest and lightest shading) and the two light chains (both in moderate, grey shading), make up the two hinged Fab domains and the one Fc domain of the IgG. Each Fab domain contains at its end an antigen binding site comprised of 6 loops of hypervariable protein chains, called complementarity-determining regions (CDRs), three from the light chain (L1-3), and three from the heavy chain (H1-3) (Adapted from Harlow and Lane (1988) and Silverton et al. (1977)).

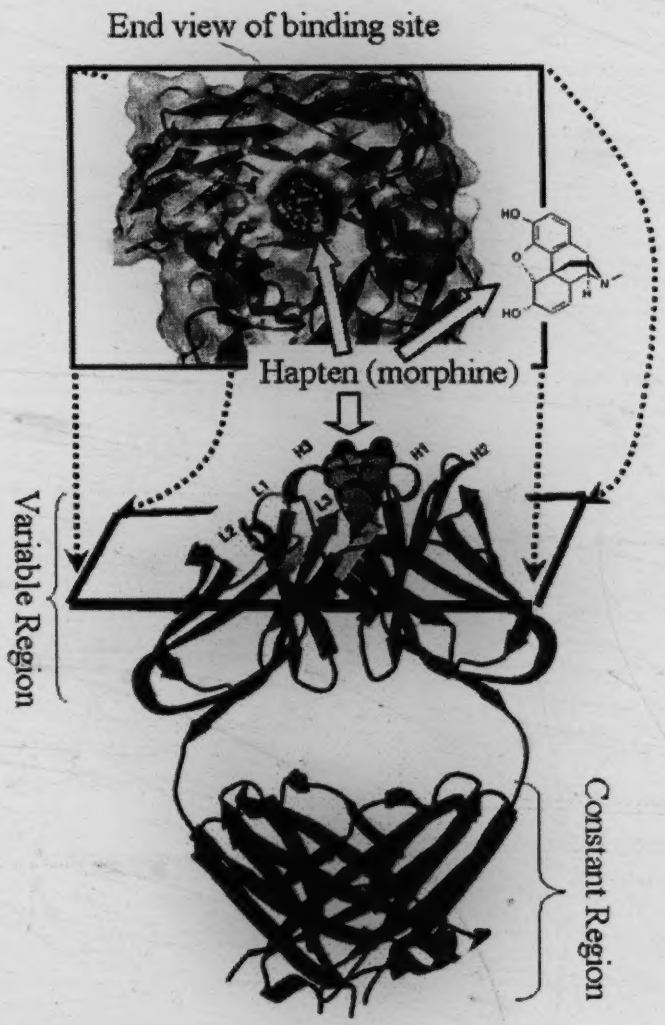


Figure 1.8. Model of an antibody binding a morphine hapten.

A ribbon representation of the folding of heavy and light chains of an antibody, forming a binding site for a hapten (morphine). The variable domain, which contains the hapten-binding site, corresponds to the N-terminal part of both chains. The six complementarity-determining regions (CDRs) responsible for interactions with the hapten are numbered L1 -3 and H1-3 for light and heavy chains. The end view of the binding site shows a pocket that intimately accommodates the hapten (Adapted from Pozharski et al. (2004)).

from the IAC. Also, if the pH of the elution buffer were decreased to as low as 2.5 (equivalent to the pK_a of the N^4-aryl amino group), sulfonamides would become partially positively charged, a state that would further accentuate their elution from the positively charged antibody binding site by electrostatic repulsion.

1.5.4. Low Column Capacities Associated with Immunoaffinity Chromatography of Small Molecules

A major limitation of immunoaffinity chromatography is that the loading capacity for small analytes is low, consequently requiring larger IAC volumes to extract a givenamount of hapten, which is then associated with larger elution volumes that often necessitate post-IAC concentration with solid phase extraction (SPE). This low capacity phenomenon exists for a hapten because the antibody - a very large macromolecule of 150,000 Da – is about 500 times more massive than the hapten it is binding (about 300 Da.). In this scenario, a limit of 10-20 mg of IgG attached per mL of hydrated solid phase gel would have a maximum hapten loading capacity of 20-40 μg/mL under ideal conditions, or 100 fold less than what a reverse-phase SPE could hold due to hydrophobic interaction in porous beads with a large surface area. The problem is further compounded when using polyclonal antibodies because only about 10-15% of the purified IgG fraction from plasma is specific to a hapten immunogen, so most polyclonal antibodies attached to an IAC are not participating in the extraction of hapten. However, with the advent of monoclonal antibody technology, the availability of pure IgG specific for a hapten has partially remedied this problem. Instead of the low level specific fraction of polyclonal antibodies, monoclonal antibodies are 100% specific.

Immunoaffinity chromatography for the purification of drugs, hormones, pesticides, and toxins is a mature technology and has been extensively reviewed (Delaunay et al., 2000; Hennion and Pichon, 2003; Pichon et al., 1997; Stevenson, 2000). Many IAC products for drugs, pesticides, and toxins are available commercially, and the literature is replete with examples of high capacity IACs using monoclonal antibodies. However, despite the associated advantages of higher affinity and diversity for hapten-classes and their metabolites, reports of using purified, hapten-specific polyclonal antibodies are rare. Most examples employing polyclonal antibodies use the unpurified

IgG fraction and consequently have low IAC hapten capacity, or even lower capacity when using unpurified whole serum. Purifying polyclonal antibodies has been fraught with difficulties in obtaining the hapten-specific fraction without denaturing it, mainly due to linker arm effects. Antibodies raised by immunizing with haptens conjugated to proteins can result in extremely high antibody affinities to hapten-linker arm complexes. Subsequently, when haptens are covalently attached to solid phase medium for use in binding and enriching hapten-specific antibodies, the bound antibodies are sometimes not able to be eluted from the hapten-linker arm complex. This is likely due to high antibody affinity to both the hapten and its associated solid phase linker arm attachment.

Purification of hapten-specific polyclonal antibodies has been reported for facilitating sulfonamide ELISA analysis (Assil et al., 1992a), but the fractional yields were extremely low (0.2% of the whole IgG population). Greater success was achieved with immunopurification of anti-methamphetamine antibodies for ELISA analysis, since they used hapten analogues different from the original immunizing hapten (Choi et al., 1997). However, aside from immunopurified polyclonal antibodies used to extract phenylurea using a similar analyte strategy (Ben Rejeb et al., 1998b), no reports using the hapten-specific fraction of polyclonal antibodies in IAC could be found.

1.6. MATRIX ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

1.6.1. History of MALDI-TOF MS

Mass spectrometry has been crucial to understanding the structure of the atom and the nature of matter, and there is no scientific field of study left untouched by its influence. Early experiments probing the nature of matter at the beginning of the twentieth century provided the basis for the development of mass spectrometry. Exploration of positive ray deflection patterns by J. J. Thompson established the existence of stable (non-radioactive) isotopes. Later, mass spectrometry played a key role in the development of atomic energy. After World War II, when mass spectrometry began to be used for the identification and quantification of organic compounds, commercially built instruments became available and were used in more diverse fields.

Mass spectrometry characterizes matter through the separation and detection of

gas-phase ions according to their mass-to-charge ratio (*m/z*). Before the 1980s, it was only used on neutral molecules or atoms that could be put into the gas phase before their ionization, which limited the application to volatile, thermally stable substances. Critical developments in "soft" ionization techniques, such as matrix-assisted laser desorption/ionization (MALDI) (Karas et al., 1987; Karas and Hillenkamp, 1988a; Takatsuki and Kikuchi, 1990) and electrospray ionization (ESI) (Yamashita and Fenn, 1984), can now drive gas phase ions from the condensed phase of a solution or a solid matrix. The introduction of the time-lag focusing concept in time-of-flight (TOF) analyzers enhanced mass resolution by correcting for the initial spatial distribution of ions, and was ideal for coupling to MALDI due to the precise timing of the ionization event. Quadrupole mass analyzer instruments were also introduced and they far exceed the total number of other types of mass spectrometers in use today. These recent advancements in mass spectrometry dominate the literature, because for the first time they allow accurate mass characterization of large, thermally labile macromolecules, such as proteins and nucleic acids, with little or no fragmentation (Guilhaus et al., 1997). Advancements in mass spectrometry, molecular biology, and computer technology are all complementary, and together are revolutionizing the potential of biological sciences into the 21st century.

1.6.2. Theory of MALDI-TOF MS

Like all mass spectrometry, MALDI-TOF MS sorts ions by mass, with the instrumentation involving an ion source to generate ions, a mass analyzer to separate ions according to their *m/z* values, and an ion detector. In MALDI-TOF MS, sample molecules embedded in an ultraviolet-absorbing matrix are ionized by a nanosecond laser pulse. During the process, highly energized ions and neutral molecules are ejected from the solid surface into the gas phase. After a short time delay, ions are accelerated toward the mass analyzer by an electric potential, separated by their mass-to-charge ratios during flight, and converted into electrical signals at the detector. A schematic diagram of the MALDI-TOF MS system is shown in Figure 1.9.

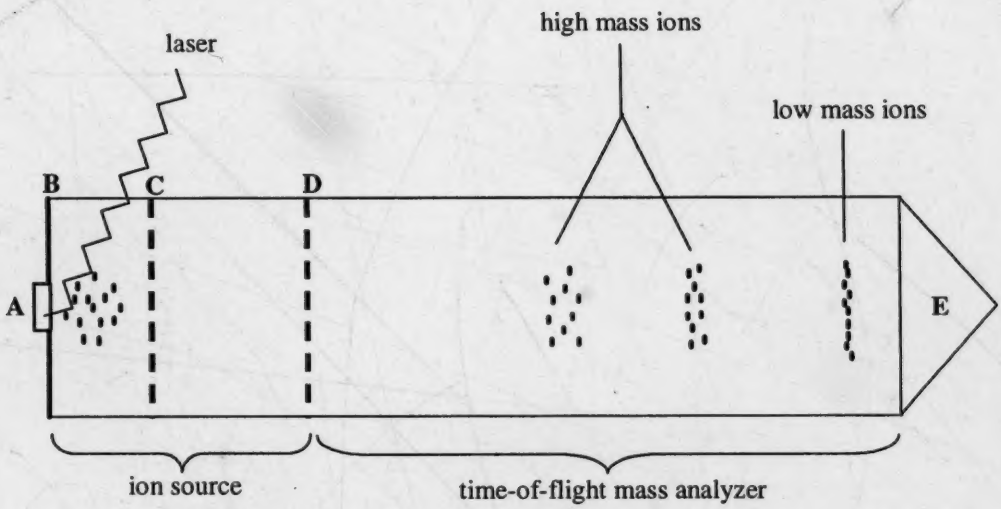


Figure 1.9. Schematic diagram of linear matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. A: sample probe tip; B: repeller plate; C: extraction grid; D: acceleration grid; E: detector. (adapted from S. Frison (2003)).

Ionization Mechanisms

Aside from facilitating the ionization process and transforming the analytes into the gas phase, the matrix protects the analytes from extensive fragmentation. Matrices employed in MALDI-TOF MS are generally small organic molecules that absorb ultraviolet light and are stable under vacuum conditions. The ion formation process in MALDI-TOF MS is complex and depends on a number of factors which are either dependent or independent of the matrix. Formation of protonated, deprotonated, cationized, and even radical species are generated relatively independently of the matrix, solvent composition, solution pH, and analyte acid-base properties (Karas et al., 2000). Although several MALDI-TOF MS ionization models imply that analyte molecules are in close contact with matrix molecules during the formation of the desorption plume (Kinsel et al., 1997; Kinsel et al., 1999; Zenobi and Knochenmuss, 1998), spectra generally do not exhibit ion signals corresponding to matrix adducts (Itina et al., 2002). Secondary

ionization steps may completely mask primary ionization by processes that convert ions by proton, cation, or electron transfer reactions, or charged particle ejection (Zenobi and Knochenmuss, 1998). Unique to the MALDI ionization process, photoionization of the matrix results in loss of electrons from the plume, resulting in the vast majority ions being singly and positively charged (Karas et al., 2000). Based on this "cluster ionization" model, highly mobile electrons are more likely to neutralize highly positively charged initial clusters, making neutralization the dominant process, and the only ions with a significant probability of surviving and being detected are the singly charged ones.

Time-of-Flight Analyser

The mass analyzer consists of a flight tube under high vacuum, which is a field-free drift region in linear instruments, and involves ion deflection by electrical fields in reflector instruments. Unlike other mass analyzers, TOF MS does not scan the spectrum, so most ions entering the flight tube are detected (Guilhaus, 1995). Initially, ions possess similar kinetic energies but different velocities, and as they travel down the flight tube they separate into discrete packets according to their mass; smaller ions travel faster and are detected before larger ions. An ion's time of flight is proportional to the square root of its *m/z* value. MALDI-TOF MS produces almost exclusively singly charged positive ions (Karas et al., 2000), unlike ESI-MS where multiple-charged ions make for a more complex spectrum.

To achieve better resolution, it was necessary to correct for the initial spatial, temporal, and velocity distribution of the ions. To attain isotopic mass resolution in a linear time-of-flight mass analyzer, a delayed extraction technique was developed and is now used in all MALDI-TOF MS instruments (Brown and Lennon, 1995; Whittal and Li, 1997). This short time delay between laser ionization and ion extraction, allows the energy correction necessary to focus the ions at the detector. The faster ions spend less time in the acceleration field, and as a consequence the slower ions have a chance to later catch up by receiving acceleration energy for a longer time. Reflectron MALDI-TOF MS analyzers have even greater resolution, because ions with the same *m/z* value accelerate into a magnetic field, where they can be focused together as they curve through the field at rates proportional to their different entry kinetic energies. Also, reflectron time-of-flight analyzers have the further advantage of being able to monitor post-ion source decay or fragmentation, which offers more structural information on the analyte.

1.6.3. Matrix Selection and Sample Preparation

The choice of matrix is crucial for success in MALDI-TOF MS experimentation. In general, MALDI-TOF MS matrices need to provide efficient ionization, controllable and reproducible fragmentation, and result in no significant spectral mass interferences with the analytes. Matrix selection is still largely an empirical process, but certain generalizations can be made in consideration of its solubility in organic solvents and miscibility with the sample solution (Nielen, 1999). Although solution dynamics may not necessarily be relevant in predicting the subsequent gas-phase interactions at the ion source, the polarity of the matrix is often matched with the polarity of the analytes to achieve a consistent and homogenous cocrystallization in whatever solvent system(s) are chosen.

Sample preparation is a key factor in the success of MALDI-TOF MS analysis. The "dried droplet" method was the original and simplest method described for MALDI-TOF MS sample preparation, and is still commonly used. It consists of mixing the analyte and matrix together in an appropriate solvent with a large molar excess of matrix, depositing the mixture onto the target probe, and removing the solvent through air-drying (Bruker Analytical Systems, 1997; Karas and Hillenkamp, 1988b). Growth of crystals occurs during solvent evaporation, and analyte molecules are ideally uniformly incorporated into the matrix crystal network. Rapidly forming, impure, smaller crystals with a homogeneous structure are desirable, so that analytes are not excluded from the matrix (Timpin et al., 2001).

In MALDI-TOF MS, the "sweet spot" phenomenon is associated with poor reproducibility due to the difficulty in evenly dispersing analytes throughout the solid crystalline matrix. This phenomenon is observed both between separate ionization events within a single spectral determination and between different spectral determinations from the same sample. Here, as the analyst is varying the laser energy to find the critical level, he or she scans across a sample spot on the probe, or even must go to a different probe spot, to locate a region which generates a signal of adequate intensity. Several techniques have been described to decrease this variation inherent with MALDI ionization. In general, they focus on generating smaller, impure crystals in homogeneous crystal beds by proper solvent selection, fast evaporation by using heat, fan, or vacuum, crystal-seeding, multi-component matrix systems, electrospray deposition, and multi-laying

strategies (Dai et al., 1999; Hensel et al., 1997; Nicola et al., 1995; Onnerfjord et al., 1999; Vorm et al., 1994; Wilkinson et al., 1997).

1.6.4. General Considerations of MALDI-TOF Analysis of Small Molecules and Specific Considerations with Respect to Sulfonamides

MALDI-TOF MS is a versatile instrument capable of analyzing either low or high molecular weight compounds. The popularity of MALDI-TOF MS is associated with its ability to measure the mass of various large, fragile, and non-volatile biomolecules up to 1.5 million Da (Schriemer and Li, 1996). However, it is equally capable of analyzing small molecules, such as small organic acids, peptides, carbohydrates, detergents, and pharmaceutical compounds (Cohen and Gusev, 2002; Goheen et al., 1997). The greatest concern when analyzing small molecules by MALDI-TOF MS is interference from matrix peaks in the low mass region below a *m/z* value of 500. High molecular weight matrices such as porphyrins have been shown to adjust for this problem because they produce peaks in the region higher than a *m/z* value of 500 (Jones et al., 1995). Ling et al. (1998) used MALDI-TOF MS to analyze pure standards of thirty different antibiotics, including 9 sulfonamides, and compared the performance of various matrices, including porphyrins and others more common such as dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA) (Figure 1.10). Although absence of low-mass ion interferences was evident with the porphyrin matrices, they found that the DHB and CHCA matrices gave greater signals, spectrum reproducibility, and resolution. It was concluded that selecting a low molecular weight matrix that did not interfere with the analyte's signal was the best option.

Analyte derivatization is another concept proposed to avoid matrix interference, where conjugation with "tag reagent" adds about 600 Da to small analytes with available amine functional groups (Lee et al., 2003). This concept has also been suggested and tested for sulfonamides using 4-acetamidobenzenesulfonyl chloride, as a means of establishing additional confirmation of the analytes identity and to increase the sulfonamide mass above the interference region associated with the matrix (Ling et al., 1998). However, such derivatization strategies would not work for sulfonamide metabolites that have had their aryl amino groups acetylated.

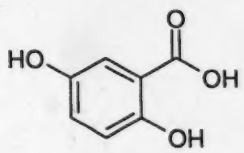
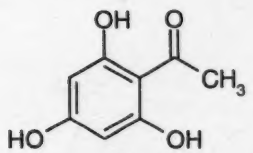
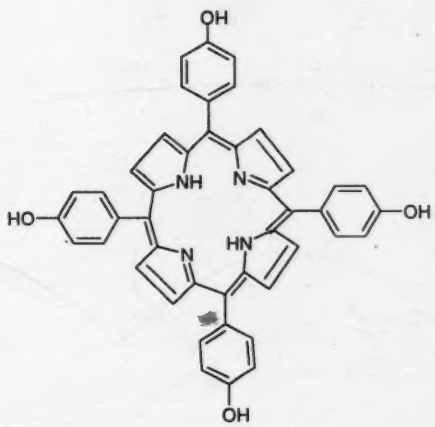
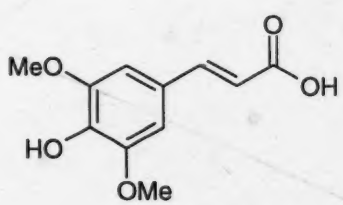
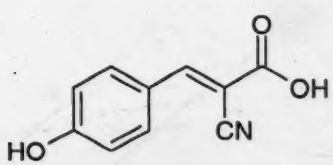
Matrix name	Structure
2,5,-dihydroxybenzoic acid (DHB)	
2',4',6'-trihydroxyacetophenone (THAP)	
5,10,15,20-tetrakis(4-hydroxyphenyl)-21H,23H-porphyrin (THPP)	
sinapinic acid (SA)	
α-cyano-4-hydroxycinnamic acid (CHCA)	

Figure 1.10. Chemical structures of common solid MALDI-TOF MS matrices.

Aside from the current research using MALDI-TOF MS to detect sulfonamides in farm environmental samples (chapters 4 and 5), the only other reported use of MALDI-TOF MS in residue analysis was to detect a glycoalkyloid fortified into pig serum (Driedger and Sporns, 2001c). Although glycoalkyloids are haptens, contrary to sulfonamides and most pharmaceutical compounds, they are large molecules (MW = 800-1000 Da) that do not experience interferences associated with MALDI-TOF MS ionization matrices.

1.6.5. Considerations in Quantitative Analysis with MALDI-TOF MS

MALDI TOF MS Sensitivity

MALDI TOF MS can exhibit high sensitivity. Femtomolar (10^{-15}) determinations are common, and as low as zeptomole (10^{-21}) quantities of large proteins have been reported (Keller and Li, 2001). Regarding sulfonamides, the limits of detection (LOD) for standard solutions was reported to be approximately 200 ng/L or about 0.6 picomole/spot when using DHB or CHCA matrices (here, LOD was defined as mean spectral baseline + 3 x analyte's standard deviation) (Ling et al., 1998). Compared to other types of mass spectrometry, MALDI-TOF MS is relatively more tolerant of low levels of buffers, salts, and some denaturants and can analyze many complex mixtures directly without extensive and elaborate chromatographic purification (Bajuk et al., 2001; Zhang et al., 2001; Zhang and Liang, 2002). Although it is capable of directly analyzing many complex mixtures, MALDI-TOF MS requires significant sample purification to analyze drug residues at ng/L levels from complex biological samples.

Quantitative Analysis with MALDI-TOF MS

The major limitation to accurate quantification with MALDI-TOF MS is the high spot-to-spot and sample-to-sample variability that results from poor crystal homogeneity and variable incorporation of analytes into the crystal bed. Strategies to minimize this variation have been described previously (section 1.6.3) regarding sample preparation and matrix selection. The most important means to compensate for the variation in MALDI-TOF MS is the use of an internal standard. Direct signal response (Petkovic et al., 2001) or external standards (Camafeita et al., 1997; Harvey, 1993; Preston et al., 1993) have been reported, however, most quantitative methods require the use of an internal standard (IS) to counteract the substantial spot-to-spot variability in MALDI-TOF MS.

The following criteria describe the ideal IS for use in MALDI-TOF MS when analyzing a small molecule (Wilkinson et al., 1997):

1. The IS must be chemically similar to the analyte so that it has similar recovery in all clean-up procedures, similar crystallization properties and incorporation into the crystalline matrix bed, and result in similar ion selection and detection in MALDI-TOF MS. An isotopically-labelled standard is ideal, but when this is not possible, then a standard as similar as possible to the analyte must be selected and verified empirically to perform similarly in the system.

2. The IS must be completely resolved from the sample and matrix peaks. Depending on the mass spectrometer, especially with the linear instruments where the resolution is relatively poor and band broadening can be a problem, more than one Dalton difference is often required between the standard and other peaks.

3. The IS must be the appropriate concentration relative to the matrix and to the analytes. Relatively high analyte concentrations can suppress the IS responses, especially for matrix-to-analyte ratios of 3000:1 (w/w) or less. The IS concentration must be enough to have a good signal-to-noise ratio to provide reliable results, yet must not be too high to suppress the analyte's response. Usually, there is a limited linear range of the analyte's response relative to the IS, and this range, usually less than 100 fold spread for a given IS concentration, can be optimized empirically by determining the best matrix concentration and conditions. When the ideal internal standard is not available empirical adjustment factors can be determined to compensate for recovery and response differences between the internal standard and the analyte(s).

Analyte Identity Confirmation

The signal of an analyte or standard is considered to be the sum of the peak heights or areas of all fragments and adducts (usually proton, sodium, and potassium adducts when the instrument is operated in the positive ion mode). Usually, the relative intensity pattern of MALDI-TOF MS spectral adducts and fragments are similar for similar compounds, such as internal standards and the analytes. The fragment masses and the patterns of the fragment and adduct peaks are consistent and predictable, and can be used to assist in confirming the identity of an analyte. The reflectron MALDI-TOF mass spectrometers can provide more information about fragmentation of analytes occurring after the initial ionization events, and ionization conditions can be adjusted to

increase fragmentation for purposes of analyte identification. Sulfonamides can fragment due to the absorption of ionization energy, and have a diagnostic fragment representing 64 Da less than the parent peak due to the excision of a sulphate moiety (SO_2).

With high resolution instruments, it is also theoretically possible to gain information about the identity of the compound by the isotopic peak intensity distribution. The sulfurs of sulfonamides naturally occur in a relative abundance of 4.3% in the ^{34}S isotope form, which when compared to carbon, oxygen, nitrogen, or hydrogen atoms, represents a relatively high proportion of an isotope two mass units greater than the main form. Using sulfathiazole as an example, the proton adduct of this sulfonamide would be expected to have three significant peaks representing a relative isotopic mass distribution of 0.804, 0.102, 0.082 for peaks at *m/z* values of 256.01, 257.01, and 258.01, respectively. On the other hand, the identity of the acetylsulfamethazine fragment (protonated and minus SO_2; *m/z* of 257.14), which has a very close *m/z* value to sulfathiazole's protonated adduct, could be differentiated in mass spectrometry because it contains no sulfur and thus would have an insignificant third isotopic peak (Figure 1.11). The isotopic mass distribution of acetylsulfamethazine, would theoretically be 0.843, 0.143, and 0.013 for peaks at *m/z* values of 257.14, 258.14, and 259.14, respectively.

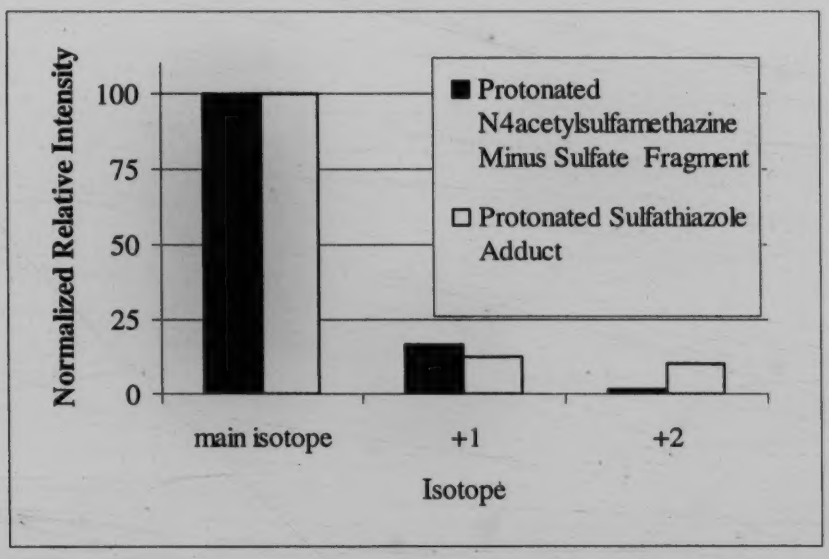


Figure 1.11. Theoretical isotopic distribution of two sulfonamide ions.

1.6.6. Considerations of Coupling, Automation, and High Throughput Technologies Associated with MALDI-TOF MS

MALDI-TOF MS naturally lends itself to high-speed analysis. Miniature arrays of samples can be applied with a piezoelectric pipet to microchip probes and analyzed in seconds per sample (Foret and Preisler, 2002b; Little et al., 1997d). Unlike electrospray ionization, which is well-suited to on-line coupling with liquid chromatography, MALDI-TOF MS is not as easily coupled to continuous on-line separation systems. Dried crystal beds combining analyte and matrix are necessary for MALDI-TOF MS analysis, and these physical requirements are not easily coupled to a continuous liquid solvent system like HPLC. Yet the dried spot preparation method can easily be prepared on mass and coupled off-line with MALDI-TOF MS.

Recent advancements in this area have focused on the potential of MALDI-TOF MS for high throughput proteomic and genetic research. Instruments are now capable of automated peptide mapping by coupling them with two dimensional gel electrophoresis systems that robotically perform protein spot excision, *in situ* proteolysis, extraction of the cleavage products from the gel matrix, and peptide purification and concentration (Gevaert and Vandekerckhove, 2000; Nordhoff et al., 2001; Traini et al., 1998). Perhaps more significant is its rapidly evolving ability in large scale detection of single-nucleotide polymorphisms (Pusch et al., 2002), and also the probability that MALDI-TOF MS will soon out-perform and replace the electrophoresis-based Sanger concept in DNA sequencing (Bocker, 2003; Chen et al., 1996; Murray, 1996). Commercial instruments like the Applied Biosystems 4700 Proteomics™ MALDI-TOF MS/MS Analyzer (Applied Biosystems, Foster City, CA) are capable of analyzing 1000 samples per hour; these developments in automated spectrum data acquisition and processing no longer make MALDI-TOF MS the limiting efficiency factor in these biological analyses (Nicola et al., 1998a).

Progress in high throughput MALDI-TOF MS macromolecular analysis is opening doors for similar improvements in other areas such as large scale combinatorial drug analysis and molecular screening studies (Hsieh et al., 1998; Nicola et al., 1996). In automated food or environmental residue analysis, MALDI-TOF MS/MS has the potential to greatly improve efficiency over current MS/MS technologies. If effective alternatives are found to the traditional, rate-limiting "bottle necks" (i.e. HPLC and GC)

in sample-handling and clean-up procedures, then residue analysis efficiency could go well beyond the current limitation of about 150 samples per day (based on the assumption of 10 min HPLC on-line cycle). Due to its higher tolerance of sample impurities, more efficient means of sample enrichment could be coupled with MALDI-TOF MS, increasing the speed of residue analysis to thousands of samples per day.

Immunoaffinity chromatography may be one of the most promising clean-up technologies capable of complementing the high throughput potential of MALDI-TOF MS. Automation and on-line coupling of immunoextraction with HPLC-MS systems is now common (Delaunay et al., 2000). The initial IAC step reduces sample matrix effects specifically and simply prior to final analyte separation and analysis. Also, the immunoextraction step offers another confirmatory dimension to the analysis of an analyte, and augments the other separation/detection parameters. However, the greatest potential in high throughput immunoextraction is not with serial, on-line clean-up processes, but rather with robotic off-line processing of samples, where parallel, miniaturized systems could simultaneously and automatically purify analytes prior to MALDI-TOF MS. Miniaturization of solid phase extraction (SPE) systems in silicon microchips is being tested for use in proteomic research in coordination with MALDI-TOF MS analysis of proteins (Ekstrom et al., 2002a). Similarly, SPE and IAC could theoretically be coordinated in tandem on microchips to rapidly extract sulfonamides or other pharmaceuticals from small samples. MALDI-TOF MS, as already stated, can ultimately process many thousands of samples per day. It is capable of accommodating very small quantities of samples, and is relatively tolerant of impurities, thereby making the purities achieved by HPLC or GC separation unnecessary. For these reasons, off-line purification technologies that efficiently prepare multi-array target probes, loaded in the MALDI-TOF MS as probe sets, may be the best target to advance MS efficiency.

Besides the current residue detection research (chapters 4 and 5), few other immunoaffinity enrichment concepts have been utilized in conjunction with MALDI-TOF MS. Following C_{18} SPE from fortified pig serum, glycoalkaloids (MW approximately 800-1000) were purified by antibody-coated agrarose beads and eluted directly onto a MALDI-TOF MS probe for detection (Driedger and Sporns, 2001b). Other examples include on-probe attachment of antibodies to a nitrocellulose-coated probe, and the subsequent on-probe extraction and analysis of a peptide (Liang et al.,

1998). Two other examples are reported that use immunoaffinity chromatography coupled to MALDI-TOF MS, but they were used in analysis of larger peptide molecules (Kawahara et al., 2002; Papac et al., 1994).

1.7. THESIS OBJECTIVES

Generally, the objective of this thesis was to improve analysis efficiency of organic residues in complex biological or environmental samples. Specifically, the research focused on the development and characterization of sulfamethazine-specific antibodies as a means to immuno-extract sulfonamide residues from environmental samples, and then to detect the enriched residues with matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The specific objectives are outlined as follows:

1. To develop sulfamethazine and sulfathiazole-specific polyclonal antibodies using azo and succinyl linking arm immunization strategies (Chapter 3).
2. To make immunoaffinity columns from the sulfonamide-specific fraction of the polyclonal antibodies candidates (Chapter 4).
3. To determine if ELISA of serum antibodies could be used to predict the subsequent performance of these antibodies in immunoaffinity chromatography (Chapters 3 and 4).
4. To use immunoaffinity chromatography to develop a rapid purification system for sulfamethazine and its acetyl metabolite (Chapters 4 and 5).
5. To detect ng/L-level sulfamethazine and its acetyl metabolite fortified in water, soil, and manure using immunoaffinity chromatography coupled with MALDI-TOF MS (Chapter 5).
6. To use the immunoaffinity-MALDI-TOF MS system to detect sulfamethazine residues in environmental samples incurred by normal agricultural practices (Chapter 6).

Chapter 2

MATERIALS AND EXPERIMENTAL METHODS

2.1. GENERAL PROCEDURES

Melting points were determined on a Gallenkamp melting point apparatus using a calibrated thermometer. Nuclear magnetic resonance (NMR) and microanalyses were performed by the University of Alberta Chemistry Services. An Inova 600 Spectrometer (Varian, Palo Alto, CA) was used to perform 1D proton, HMQC proton-carbon correlation, and APT (attached proton test) experiments on sulfonamide derivatives dissolved in DMSO-d_6. A Perkin-Elmer 240 CHN analyzer was used for carbon, hydrogen and nitrogen. Sulfur was determined using barium perchlorate titration after the sample had been burnt in an oxygen filled flask.

2.2. REAGENTS AND BUFFERS

All chemicals were at least reagent grade, and all reagent water used in this research was deionized by a Milli-Q purification system (Millipore Corp., Bedford, MA). Tris(hydroxymethyl)aminomethane hydrochloride (TRIS), ammonium bicarbonate, ammonium sulfate, ammonium sulfamate, sodium periodate, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB), sodium nitrite, sodium azide, Tween 20, N-chloroacetyl-L-tyrosine (NCATyr), N-α-acetyl-L-histidine (NAHis), bovine serum albumin (BSA), *Limulus polyphemus* hemolymph (LPH), and the following sulfonamides were obtained from Sigma Chemical Company (St. Louis, MO): sulfamethazine (SMT), sulfamerazine (SMR), sulfathiazole (STZ), sulfanilamide (SF), sulfadiazine (SDA), sulfapyradine (SPD), sulfadimethoxine (SDM), and succinylsulfathiazole (Succinyl-STZ). Acetic anhydride was obtained from Caledon Laboratories (Georgetown, ON). Hyflo Super Cel celite, washed sea sand, 2,5-dihydroxybenenzoic acid (DHB), sodium chloride, sodium phosphate, citric acid, sodium acetate, glycine, glacial acetic acid, n-hexane, ethyl acetate, glycerol, methanol, and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Ottawa, ON). Anhydrous ethanol was obtained from Commercial Alcohols (Winnipeg, MB). Succinic anhydride was supplied by BDH Chemicals (Toronto, ONT). N-1-napthylethylenediamine dihydrochloride (NED) was supplied by MCB Manufacturing Chemists, Inc (Cincinnati, OH). Freund's complete and Freund's incomplete adjuvants were obtained from DIFCO Laboratories (Detroit, MI). Urea peroxide and goat anti-

rabbit peroxidase-conjugated antibodies (GAR-HRP) were supplied by Calbiochem Co. (San Diego, CA). Commonly used buffers and reagents are described in Table 2.1.

2.3. PREPARATION OF SULFONAMIDES

2.3.1. Parent Sulfonamides

Commercially obtained sulfonamides were recrystallized from a 10% (w/v) solution in DMSO/water (1:1 v/v), washed extensively with water, dried for 24 hr at 100 °C in a forced air oven, and stored in a desiccator.

Sulfamethazine

Melting point = 197-200 °C. MALDI-TOF MS analysis confirmed the expected peaks at m/z 279.1, 301.1, 317.1, 215.1 for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions. ^{1}H NMR (600 MHz, $DMSOd_6$) δ 11.05 (br s, 1 H, sulfonamide NH), δ 7.64 (d, 2 H, 9 Hz, ArH), δ6.72 (s, 1 H, ArH), δ 6.55 (d, 2H, 9 Hz, ArH), δ 5.95 (s, 2 H, $ArNH_2$), δ 2.22 (s, 6H, CH_3).

Sulfathiazole

Melting point = 200-203 °C. MALDI-TOF MS analysis confirmed the expected peaks at m/z 256.0, 278.0, 294.0, 192.1 for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions. ^{1}H NMR (600 MHz, $DMSOd_6$) δ 11.40 (br s, 1 H, sulfonamide NH), δ 7.42 (d, 2 H, 9 Hz, ArH), δ 7.17 (d, 1 H, 4.5 Hz, thiazole H), δ 6.73 (d, 1 H, 4.5 Hz, thiazole H), δ 6.57 (d, 2 H, 9 Hz, ArH), δ 5.90 (s, 2 H, $ArNH_2$).

2.3.2. N^4-Acyl Derivatives of Sulfonamides

The synthesis procedure is shown in Figure 2.3, and has been described in the literature for the acetyl derivative but not for the succinyl or propionyl derivatives of a sulfonamide (Whelpton et al., 1981). N^4-acetyl and N^4-propionyl derivatives for SMT and STZ were made by reacting a ten fold molar excess of acetic anhydride and acetic acid (4 mL of 1:1 (v/v)) or propionic anhydride and propionic acid (5 mL of 1:1 (v/v)) together with the respective sulfonamide (1 g) at 100 °C for 10 min. This procedure was modified to make the N^4-succinyl derivative of SMT. Succinic anhydride and SMT were mixed with 5 mL DMSO in a 10:1 molar ratio (3.6 g succinic anhydride and 1 g SMT),

Table 2.1. Commonly used reagents and buffers

Reagent or buffer name	Ingredients	Comments
PBS (Phosphate Buffered Saline)	0.05 M sodium phosphate, 0.9 % NaCl, pH 7.2	Buffer used for coating ELISA plates, for addition of antibodies or sulfonamides onto immunoaffinity columns.
PBST (Phosphate Buffered Saline with 0.05% Tween 20)	0.05 M sodium phosphate, 0.9 % NaCl, pH 7.2 + 0.05% (w/v) Tween 20	Buffer used to dilute antibodies and standards or samples tested in ELISA
TMB substrate	3,3',5,5'-tetramethylbenzidine (TMB) at 0.6 mg/mL in acetate-citrate-urea hydrogen peroxide buffer, pH 4.0	Substrate used in ELISA. TMB stock solution (100 mg/mL DMSO) was dissolved to 0.6 mg/mL using a buffer made of 8.2 g sodium acetate per L, with enough citric acid added to adjust the solution to pH 4.0 (approx. 2.5 g), and then 150 mg urea hydrogen peroxide.
Elution buffer for antibody immunoaffinity columns (IACs)	10 mM HCl pH 2.5 or 10 mM HCl pH 2.5 and ethanol 8:2	
Elution buffer for purifying antibodies on sulfonamide-BSA IAC	0.1 M glycine pH 2.7	
DHB Matrix for matrix assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS)	2,5-dihydroxybenzoic acid dissolved (DHB) in 50% aqueous ethanol at 10 mg/mL.	Standards were mixed 1:1 with the DHB solution, or alternatively were processed through micro HLB solid phase extraction, dried under N_2(g), and redissolved with 10 µL of DHB solution. Approximately 1 µL of a matrix mixture was applied to a MALDI-TOF MS target probe.

and then dissolved and reacted by stirring and heating for 15 min in a boiling water bath. Ten mL water acidified with 1 mL concentrated HCl was added to precipitate the succinyl derivative, whereas the N^4-acetyl and N^4-propionyl derivatives crystallized spontaneously upon cooling. The resulting crystals were collected by vacuum filtration through sintered glass funnels and washed extensively with water. Derivatives were recrystallized from a 10% (w/v) solution DMSO/water (1:1 v/v), washed extensively with water, dried for 24 hr at 100 °C in a forced air oven, and stored in a desiccator.

N^4-Acetylsulfamethazine

Melting point = 248-250 °C. MALDI-TOF MS analysis confirmed the expected peaks at m/z 321.1, 343.3, 359.1, and 257.1 for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions. The carbon, hydrogen, and nitrogen content were 52.71%, 4.82%, and 17.21%, respectively (expected theoretical values are 52.49%, 5.07%, and 17.50%). ^{1}H NMR (600 MHz, $DMSOd_6$) was consistent with the structure of NA-SMT: δ 11.53 (br s, 1 H, sulfonamide NH), δ 10.21 (s, 1 H, ArNH), δ7.89 (d, 2 H, 9 Hz, ArH), δ 7.68 (d, 2H, 9 Hz, ArH), δ 6.65 (s, 1 H, ArH), δ 2.21 (s, 6 H, CH3), δ 2.01 (s, 3 H, CH_3). HMQC proton-carbon correlation and APT (attached proton test) experiments on this derivative were consistent with the structure of NA-SMT.

N^4-Propionylsulfamethazine

Melting point = 219-221 °C. MALDI-TOF MS analysis confirmed the expected peaks at m/z 335.1, 357.1, 373.1, and 271.2 for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions. ^{1}H NMR (600 MHz, $DMSOd_6$) was consistent with the structure of NP-SMT: δ 11.58 (br s, 1 H, sulfonamide NH), δ 10.20 (s, 1 H, ArNH), δ 7.89 (d, 2 H, 9 Hz, ArH), δ 7.68 (d, 2 H, 9 Hz, ArH), δ 6.73 (s, 1 H, ArH), δ 2.33 (q, 2H, 7.5 Hz, alkyl CH_2), δ 2.23 (s, 6 H, $ArCH_3$), δ 1.05 (t, 3 H, 7.5 Hz, alkyl CH_3).

N^4-Succinylsulfamethazine

Melting point 237-240 °C. MALDI-TOF MS analysis confirmed the expected peaks at m/z 379.1, 401.1, 423.1, and 315.1 for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions. The carbon, hydrogen, and nitrogen content were 44.41%, 3.57%, 13.64%, respectively (expected theoretical values are 44.44%, 3.73%, and 13.64%, respectively). ^{1}H NMR (400 MHz, $DMSOd_6$) was consistent with the structure of Succinyl-SMT: δ 11.82 (br s, 2 H, sulfonamide NH and ArNH), δ 10.25 (s, 1 H, COOH), δ 7.87 (d, 2H, 9Hz, ArH), δ

7.67 (d, 2 H, 9 Hz, ArH), 6.70 (s, 1 H, ArH), δ 2.52 (t, 2H, 7 Hz, alkyl CH_2), δ 2.48 (t, 2H, 7 Hz, alkyl CH_2).

N^4-Acetylsulfathiazole

Melting point = 248-252 °C. MALDI-TOF MS analysis confirmed the expected peaks at m/z 298.0, 320.0, 336.0, 234.1 for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions. ^1H NMR (600 MHz, $DMSOd_6$) was consistent with the structure of NA-SMT: δ 12.61 (br s, 1 H, sulfonamide NH), δ 10.21 (s, 1 H, ArNH), δ 7.73 (d, 2H, 9 Hz, ArH), δ 7.69 (d, 2H, 9 Hz, ArH), δ 7.22 (d, 1 H, 4.5 Hz, thiazole H), δ 6.79 (d, 1 H, 4.5 Hz, thiazole H), δ 2.04 (s, 3 H, CH_3). HMQC proton-carbon correlation and APT (attached proton test) experiments on this derivative were consistent with the structure of NA-STZ.

N^4-Propionylsulfathiazole

Melting point = 249-251 °C. MALDI-TOF MS analysis confirmed the expected peaks at m/z 312.0, 334.0, 350.0, 248.1 for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions. ^1H NMR (600 MHz, $DMSOd_6$) was consistent with the structure of NP-STZ: δ 12.63 (br s, 1H, sulfonamide NH), δ 10.19 (s, 1 H, ArNH), δ 7.66 (s, 4 H, ArH), δ 7.22 (d, 1 H, 4.5 Hz, thiazole H), δ 6.79 (d, 1 H, 4.5 Hz, thiazole H), δ 2.33 (q, 2 H, alkyl CH_2), δ 1.06 (t, 3 H, alkyl CH_3).

N^4-Succinylsulfathiazole (commercially obtained)

Melting point 184-194 °C. MALDI-TOF MS analysis confirmed the expected peaks at m/z 356.0, 378.0, 394.0, and 292.1 for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions. ^1H NMR (400 MHz, $DMSOd_6$) was consistent with the structure of Succinyl-STZ: δ 12.4 (br s, 2H, sulfonamide NH and ArNH), δ 10.3 (s, 1H, COOH), δ 7.70 (s, 4H, ArH), δ 7.22 (d, 1H, 4.5 Hz, thiazole H), δ 6.79 (d, 1H, 4.5 Hz, thiazole H), δ 2.57 (t, 2 H, 6 Hz, alkyl CH_2), δ 2.51 (t, 2H, 6 Hz, alkyl CH_2).

2.3.3. *N^4-Azo*-Amino Acid Derivatives of Sulfonamides

N-Chloroacetyl-L-tyrosine sulfamethazine (NCATyr-SMT), N-chloroacetyl-L-tyrosine sulfathiazole (NCATyr-STZ), N-α-acetyl-L-histidine sulfamethazine (NAHis-SMT), and N-α-acetyl-L-histidine sulfathiazole (NAHis-STZ) were synthesized. Amino acid sulfonamide derivatives were synthesized as ELISA competitor ligands and also as standards to determine hapten-protein molar incorporation ratios using the diazo reaction

(Garden and Sporns, 1994). These derivatives were made by weighing 24.4 mg SMT or 22.8 mg STZ, and dissolving each in 10 mL of 0.1 N NaOH. Then, the following reagents are added in order and mixed after each addition: 0.75 mL parent sulfonamide solution or 0.75 mL 0.1 N NaOH blank, 0.75 mL 3.5 M HCl, 0.23 mL of 1% (w/v) sodium nitrite, and 0.35 mL of a 0.8% aqueous solution of ammonium sulfamate, 0.75 mL of N-chloroacetyl-L-tyrosine solution (13.52 mg per mL of 0.1 N NaOH) or N-α-acetyl-L-histidine (10.34 mg per mL 0.1 N NaOH), and 0.75 mL 6 N NaOH. The solutions stirred overnight at 4 °C. These amino acid derivatives were difficult to recrystallize, so they were used without further purification assuming the reactions had gone to completion. Completion of the reaction was supported by the observation that there were no observable MALDI-TOF MS peaks associated with the parent sulfonamides in the product. Experimental effects of the other reagents contaminating amino acid-azo-sulfonamide standards were shown to be negligible when testing the reagent blank controls.

2.3.4. Standard Sulfonamide Solutions

Stock Sulfonamide Solutions

Stock solutions were made by accurately weighing 100 mg of each powder to the nearest milligram, dissolving it in DMSO, and then making it up to 10.00 mL in a volumetric flask with DMSO. These 10,000 µg/L DMSO stocks were stored in the dark at room temperature (RT), and fresh stocks were made after 4 months storage.

The only sulfonamide stock solutions that were not made in this manner were the NCATyr and NAHis amino acid derivatives of SMT and STZ because they could not be crystallized. Their synthesis reactions were assumed to go to completion, so the moles produced of each derivative was assumed to be equivalent to the limiting sulfonamide mole amount added to each reaction. After the reaction mixtures had been stirred overnight at 4 °C, the solutions were adjusted to pH 7.5 with 3.5 M HCl, then transferred to 10.00 mL volumetric flasks, and made up to the marks with PBS. The final concentrations of these stocks were therefore 658 µM for the SMT amino acid derivatives (24.4 mg SMT added to the reaction/10 mL final volume) and 671 µM for the STZ amino

acid derivatives (22.8 mg STZ added to the reaction/10 mL final volume). Aliquots of these stocks were frozen at -25 °C and thawed as required.

Standard Sulfonamides for Bratton-Marshall (BM) Assay, ELISA, and MALDI-TOF MS

Stocks for sulfonamides or their derivatives were diluted into buffer for each experiment that required working sulfonamide solutions. If less than 10 µL of stock were required to make a given sulfonamide working standard, then a 1000 µg/L intermediate stock was made by diluting the 10,000 µg/L standard by ten (100 µL stock + 900 µL DMSO). The dilution buffer used depended upon the experiment. For the BM assay, the dilution buffer was the same as the buffer containing the unknown sulfonamides being analyzed. For MALDI-TOF MS, intermediate 360 µM sulfonamide stocks were made in DMSO, and the appropriate volume of these intermediate stocks were fortified directly into the buffer or sample that was being examined at any given stage of SPIE analysis (pH 5.5 water for first stage of SPIE, 20% methanol in PBS for IAC, and 10 mM HCl or PBS for micro-HLB), or directly into ethanol for direct MALDI-TOF determination of relative response ratios. For ELISA or IAC affinity experiments, PBST + 1% BSA was used. When serial dilutions were required for ELISA (usually a serially dilution factor of 3, 4 or 5, depending on the range of sulfonamide concentrations necessary to examine in one plate), they were made directly in the ELISA plate from the top standard. The standards with the highest concentrations were made so they were equivalent molarity. These top standards were then added to column 12 wells of the ELISA plate at a volume of 100 µL + a transfer volume (100 µL + transfer volume (µL) = transfer volume x serial dilution factor). PBST + 1% BSA was added to all the other wells at 100 µL/well, and using a multi-channel pipetor the transfer volumes were taken from column 12 to column 11 wells. These same volumes were mixed by aspiration and ejection four times in column 11 wells using the multi-channel pipetor. Next using the same pipet tips, the same volumes were transferred again, this time from column 11 to 10 wells, followed by mixing, and so on down the plate to column 3. After mixing in column 3, the transfer volumes were discarded, and column 1 and 2 wells were left without competitor sulfonamide addition, and acted as blanks.

2.4. OTHER SYNTHETIC REACTIONS AND MATERIAL PREPARATIONS

2.4.1. Preparation of Sulfonamide Hapten-Protein Conjugates

Sulfonamide-protein conjugates joined by a succinyl or an azo linker arm were both prepared and their molar incorporation rates evaluated as previously described for sulfamerazine (Garden and Sporns, 1994), and were used for rabbit immunization, the making of sulfonamide-specific antibody immunoaffinity columns, or as competitor molecules in ELISA.

Succinyl-Linked Conjugates

To make the succinyl-linked sulfonamide conjugates of LPH or BSA, the succinylsulfonamides of STZ and SMT were required. Succinyl-SMT was not commercially available, and was prepared by dissolving SMT (1.0 g) and succinic anhydride (3.6g) in 5.0 mL DMSO in a 25 mL round bottom flask, and heated in a boiling water bath for 15 min. Water (10.0 mL) was added and the reaction flask cooled to 4 °C for 12 hr. The crystals that formed upon cooling were captured and washed extensively with water in a sintered glass funnel. Succinyl-SMT was recrystallized from a 10% (w/v) solution in 1:1 DMSO/water (1:1 v/v), and were again washed extensively with water, dried for 24 hr at 100 °C in a forced air oven, and stored in a desiccator. Melting points were determined, and MALDI-TOF MS and NMR were performed to confirm the identity and purity of both succinylsulfonamides (succinyl-SMT and succinyl-STZ), and microanalysis was performed on succinyl-SMT. The production of succinyl-linked sulfonamide conjugates involved reacting succinylsulfonamide with the primary amine of lysine amino acids of a protein. The synthesis of succinyl-linked conjugates attempted to achieve a "moderate loading", or a 10 to 20 molar sulfonamide to protein incorporation ratio (Garden and Sporns, 1994). Succinyl-SMT or Succinyl-STZ (0.055 mmol, or approximately 20 mg) and (EDC) (0.11 mmol or 21 mg) were dissolved in 1.0 mL anhydrous DMF, and this mixture was added dropwise to a rapidly stirring solution composed of 50 mg of BSA or LPH (approximately $8x10^{-4}$ and $7x10^{-4}$ mmol, respectively) dissolved in 1 mL water made basic with 20 µL of 6N NaOH in a 4 mL vial. After the solution was allowed to react for 6 hr at RT, 100 µL of 21 mg/mL EDC was added dropwise, and the system was allowed to react for another 12 hr. Next, the reaction contents were transferred to Spectra/Por 2 membrane tubing (MW cutoff of 12-

14 kDa) with washings of 8 M urea, and dialyzed against 1L 8 M urea for 4 hr, and then dialyzed again with fresh 8 M urea for a further 12 hr. The dialysis tubing and contents were then transferred to 4 L of 50 mM ammonium bicarbonate and dialyzed over 6 h, and then dialyzed for a further 6 h against a fresh 4 L 50 mM ammonium bicarbonate, and then against 4 L of 25 mM ammonium bicarbonate for 12 hr. The conjugates were then removed from the dialysis tubing and freeze-dried. Problems encountered with gel formation during the reaction and with later insolubility of the lyophilized conjugates made the LPH conjugates only suitable for rabbit immunization. To make water-soluble sulfonamide succinyl-linked BSA conjugates capable of being used for ELISA, the procedure was modified by increasing the reaction mass of BSA (75 mg), reducing the mass of EDC added (11.0 mg), and limiting the reaction time to 40 min. before dialysis was initiated in 8 M urea. The conjugates were then dialyzed and lyophilized as described for the first succinyl-linked conjugates.

Azo-Linked Conjugates

The synthesis of azo-linked conjugates involved the production of a sulfonamide-diazonium ion, which reacts with a protein to form an azo-linked conjugate mainly via histidine amino acids in a protein (Garden and Sporns, 1994). SMT (7.4 g) or STZ (6.8 g) were dissolved in 1.0 mL of 3.5 N HCl and 0.4 mL of 1% (w/v) sodium nitrite was added, mixed, and reacted for 3 min at RT to form the orange-coloured diazonium ion. Then 19.6 mg of ammonium sulfamate was mixed in and allowed to react for 2 min before the solution was added dropwise while mixing to either 107 mg of LPH dissolved in 4 mL 0.1 M sodium carbonate or 260 mg of BSA dissolved in 4 mL PBS in a 25 mL Erlenmeyer flask. After 5 min, 20 drops of 6N NaOH were added, and the reaction mixture was stirred for 12 hr at 4°C in the dark. Conjugates were then transferred to Spectra/Por 2 membrane tubing (12-14 kDa cutoff) with washings of 8 M urea, and were dialyzed and lyophilized as already described for the succinyl-linked conjugates.

2.4.2. Determination of Sulfonamide -Protein Conjugate Molar Ratios

Azo-Linked Conjugate Incorporation Rate Determination

Sulfonamide incorporation rates for all azo-linked conjugates were determined based on absorbance at 430 nm according to the method described by Garden and Sporns (1994). Conjugates were prepared in 0.6N NaOH in 10 mL volumetric flasks (1.00

mg/mL), and duplicate absorbance measurements were made of these solutions at 430 nm. Based on Equation 2.1 the average absorbencies were used to calculate the molar incorporations for SMT and STZ into LPH and BSA (see appendices in Chapter 9, Table 9.2, for molar incorporation rates).

Equation 2.1. Molar sulfonamide incorporation (moles sulfonamide/mole protein)

$= A^* B^* C$

A = Absorbance at A_{430}

B = extinction coefficient of 0.123 cm^{-1}

C = optical path length (1 cm)

Succinyl-Linked Conjugate Incorporation Rate Determination

The succinyl-linked conjugates were hydrolyzed and the released sulfonamide was quantified colorimetrically using the Bratton-Marshall (BM) assay to determine the hapten-protein molar ratio. A freeze-dried conjugate sample of about 5 mg was accurately weighed into a 4 mL glass reaction vial with a Teflon-lined screw-cap and 20 µL DMSO and 2 N NaOH were added. Water was substituted for 2 N NaOH to establish controls testing for sulfonamide associated with the conjugates that was not covalently attached, and were found to be negative. Sulfonamide standards (20µL of 10.0 µg/L of SMT or STZ in DMSO) and DMSO blanks were added to reaction vials with 2 N NaOH. Conjugates and standards were sealed in their vials and put into a boiling water bath for 30 min., and then allowed to cool. Ethanol (2.0 mL) was added to each vial and mixed, and then triplicate aliquots of conjugates or sulfonamide standards (10 µL and 50 µL volumes) were analyzed using the BM assay. Samples with highest absorbencies less than 2.0 were used to determine the average mass of sulfonamide in the conjugates based on the extinction coefficient calculated from the standards. Molar incorporation of sulfonamide into BSA or LPH proteins was calculated based on Equation 2.2.

Equation 2.2. Molar incorporation = A*B/(C-A)*D

A = mass of sulfonamide determined by BM assay

B = MW of protein (MW's of BSA and LPH = 66 and 70 kDa, respectively)

C = mass of conjugate weighed

D = MW of sulfonamide (MW of STZ and SMT = 256 and 278 Da, respectively).

(See appendices, Chapter 9, Table 9.;2, for molar incorporation rates).

2.4.3. Preparation of Antibodies and Immunoaffinity Columns (IACs)

Immunizations with Sulfonamide-Limulus polyphemus Hemolymph

All animals were obtained through and maintained by the University of Alberta Animal Services, Biological Sciences Department. Rabbits were male Flemish Giant/Lop Ear crosses, 4-6 weeks old. Polyclonal antisera against diazo-conjugates of STZ-LPH and SMT-LPH were produced by immunizing rabbits with sulfonamide-*Limulus polyphemus* hemolymph conjugate. Each sulfonamide-LPH conjugate (1.0 mg) was mixed with 1 mL of sterile-filtered PBS, and then combined with 1 mL of Freund's Complete adjuvant in a water-in-oil emulsion and injected intramuscularly into a rabbit. For each rabbit each subsequent injection was made the same as the first, except with Freund's Incomplete adjuvant instead of Freund's Complete adjuvant. Rabbits were immunized every 30 days, and small venous blood samples were taken just before the first injection and 15 days after each subsequent injection to monitor the development of the antibody titre. After 3 or 4 months, once the antibody titre was not increasing further, the rabbits were sacrificed and blood was obtained by cardiac puncture. All blood samples were allowed to clot for 4 h at RT, and then for 12 hr at 4°C. The sera were decanted from the blood clots after centrifugation at 2500 X G, and then stored frozen at -20°C until used. When required, serum was thawed quickly in warm water of approximately 40°C.

Preparation of Sulfonamide-BSA Affinity Chromatography Column for Antibody Purification

Lyophilized SMT or STZ azo-linked BSA conjugate (15 mg) was reacted with 5.0 mL of swollen CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) in 0.1 M $NaCO_3$ pH 8.3 + 0.5 M NaCl (coupling buffer), and conditioned with 0.1 M TRIS pH 8.0 and 0.1 M sodium acetate pH 4.0, as recommended by the manufacturer. The sulfonamide-BSA affinity chromatography gel was equilibrated to phosphate buffered saline (PBS) + 0.05% (w/v) sodium azide and stored at 4 °C in a glass vial. When required for immuno-extraction of antibodies, the SMT-BSA or STZ-BSA affinity gel was transferred to a 5 mL pipet tip fitted with a 2 mm x 1/8" OD

frit (Supelco, Bellefonte, PA) wedged in the tip (Figure 4.1), and washed with 10 column volumes of elution buffer, and then equilibrated to PBS. Columns were regenerated between uses by washing with 10 column volumes of elution buffer, and then equilibrated to PBS.

Purification of the Sulfonamide-Specific Fraction of Antibodies by IAC

The IgG fraction of immunoglobulins from immunized rabbit sera were first purified by ammonium sulfate precipitation or by affinity chromatography using Protein G sepharose (HiTrap Amersham Pharmacia Biotech, Uppsala, Sweden). Next the sulfonamide-specific fraction of antibodies was purified using azo-linked sulfonamide-BSA affinity chromatography. For the precipitation method, an equal volume of 4°C-saturated ammonium sulfate solution was added slowly to rabbit serum while rapidly stirring, and then let mix for 12 h before centrifuging at 5,000 X g for 25 min. The supernatant was discarded and the pellet was dissolved in a minimum volume of PBS required, placed into Spectra/Por 2 membrane tubing (12-14 kDa cutoff) with washings of PBS, and then dialyzed against PBS with several changes of buffer. Alternatively, for Protein-G purification, the protocol recommended by the manufacturer was used, where antibodies are bound to a 1 mL Protein-G column in a 20 mM sodium phosphate pH 7 coupling buffer. Antibodies were then eluted with 0.1 M glycine (pH 2.7 buffer), and 1 mL fractions were collected into 25 µL 1M TRIS buffer (pH 9.0) to neutralize the pH. Fractions with an A_{280} of greater than 0.3 were pooled and then dialyzed against PBS with several changes of buffer.

To enrich the sulfonamide-specific immunoglobin fraction, batches of ammonium sulphate or Protein G-purified antibodies were applied by gravity to a 5 mL SMT-azo-BSA or STZ-azo-BSA column previously equilibrated to PBS. The columns were washed with ten column volumes of PBS, and immunoglobulins were eluted using 0.1 M glycine-HCl buffer (pH 2.7) and 1.0 mL fractions were collected into test tubes containing 25 µL of 1.0 M TRIS buffer at pH 9.0. Flow-through fractions were re-applied and eluted from regenerated sulfonamide-azo-BSA affinity chromatography columns repeatedly until no protein could be eluted from the columns. For a given batch the elution fractions having an $A_{280} > 0.3$ were pooled and then the antibodies were precipitated with ammonium sulfate (50% saturated solution at 4 °C) and stirred 12 hr at

4 °C. The resulting suspension was centrifuged at 5,000 x *g* for 30 min, the supernatant discarded, and the pellet resuspended to a concentration of 5-10 mg/mL and then dialyzed using an appropriate buffer depending on how the antibody would be used next (PBS for testing in ELISA, 0.1 M $NaCO_3$ pH 8.3 + 0.5 M NaCl for the CNBr coupling reaction of the Sepharose support, or 0.02 M sodium acetate, pH 5.0 for the hydrazine coupling reaction of the HZ support). These resuspended antibodies were placed into Spectra/Por 2 membrane tubing (12-14kDa cutoff) with buffer washings, and then dialyzed against several buffer changes. Immunoglobin concentrations in aqueous solutions were determined by UV colorimetry at 280 nm relative to the solvent buffer blank. The absorbance was converted to the concentration of IgG antibodies using an extinction coefficient of 1.35 mL/mg cm (Harlow and Lane, 1988).

Production of Anti-Sulfonamide IAC using CNBr-Activated Sepharose 4B

The binding of purified anti-SMT or anti-STZ antibodies to CNBr-activated Sepharose was carried out as recommended by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). Approximately 10 mg of antibody in 5 mL coupling buffer (0.1 M $NaCO_3$ pH 8.3 + 0.5 M NaCl) was reacted per mL of the swollen CNBr-activated Sepharose 4B gel. Incorporation of the antibody into the gel was monitored by measuring the A_{280} of the supernatant over the gel suspension. Antibody incorporation rates (Equation 2.3a) into the gels were measured by determining the unreacted antibody by UV spectrometry from a centrifuged sample of supernatant, and comparing it to the amount of antibody originally added. Percent of the reacted antibody that incorporated into the IAC was calculated by Equation 2.3b.

Equation 2.3a. Antibody incorporation rate (mg antibody/mL gel) = (A-B)/C

A = antibody added to reaction (mg)

B = antibody in supernatant after reaction (mg)

C = volume of IAC gel reacted (mL)

Equation 2.3b. Percent of reacted antibody incorporated into IAC = 100*(A-B)/A

A and B are the same as for Equation 2.3a.

The gel was placed into a 5 mL fritted glass column and conditioned with 0.1 M TRIS buffer, pH 8 for 2 h to allow the unreacted CNBr groups to be capped. Then the gel was conditioned with 3 alternating washing cycles of 0.1 M TRIS pH 8 + 0.5 M NaCl followed by 0.1 M acetate buffer pH 4 + 0.5 M NaCl to remove proteins that were not covalently attached. The anti-sulfonamide affinity gel was then equilibrated to PBS + 0.05% (w/v) sodium azide and stored at 4 °C in a glass vial. When required for immuno-extraction of a sulfonamide, enough anti-sulfonamide immunoaffinity gel was applied to a calibrated 5 mL column to give approximately 0.4 mL settled volume, and equilibrated to PBS. Buffer and samples were applied to the columns at a rate of approximately 1 mL/min by vacuum using a manifold system (Supelco, Bellefonte, PA) capable of handling 12 columns.

Production of Anti-Sulfonamide IAC using Affi-Prep Hz Hydrazide Support

The binding of purified anti-SMT or anti-STZ antibodies to Affi-Prep Hz Hydrazide Support was carried out as recommended by the manufacturer (Biorad Laboratories, Hercules, CA). In a glass vial, approximately 10 mg of immunopurified antibody in 4 mL oxidation buffer (0.02 M sodium acetate, pH 5.0) was reacted with 80 µL of 0.5 M $NaIO_4$, and then the vial was sealed, covered with aluminum foil, and gently mixed for 60 min. To stop the oxidation reaction, 200 µL glycerol was added and mixed for 10 min. Ammonium sulfate (1.8 g) was added slowly over 20 min while mixing to precipitate the oxidized antibodies. The antibodies were separated by centrifugation at 10,000 G for 5 min, and the supernatant was discarded. The pellet was resuspended in 2.0 mL coupling buffer (0.1 M sodium acetate + 1.0 M NaCl; pH 4.5). These resuspended antibodies were placed into Spectra/Por 2 membrane tubing (12-14 kDa cutoff) with buffer washings, and then dialyzed against several changes of coupling buffer for 6 hr. The Affi-Prep Hz support was supplied in isopropanol, and was prepared for coupling by washed it extensively with water and coupling buffer using a sintered glass funnel. The support was then resuspended in coupling buffer, placed in a graduated cylinder, so that the settled support volume could be estimated. Approximately 1.0 mL settled support equivalents and the antibody dialysate were added together in a sealed glass vial, covered with tin foil, and mixed for 12 hr using a rotary mixer at 10 cycles/min. Antibody incorporation into the Affi-Prep Hz Hydrazide Support, IAC conditioning,

storage, and preparation before IAC evaluation were the same as described in the previous section.

2.5. IAC PERFORMANCE CALCULATIONS

2.5.1. Theoretical IAC Binding Capacity

The theoretical binding capacities of IAC were based on the assumptions that the antibody bound to IAC is pure IgG, each antibody has two binding sites that were available for binding the sulfonamide, and that the average molecular weight of the antibody (IgG) was 150 kDa. Based on the above assumptions. Equations 2.4a and 2.4b determine the theoretical maximum specific binding performance of an IAC as sulfonamide mass (nmol) or percent of maximum, respectively.

Equation 2.4a. Theoretical maximum IAC sulfonamide binding (nmol) = $A*B*10^6/C$

A = IgG incorporation rate into IAC (mg/mL)

B = 2 sulfonamide binding sites/IgG; C = IgG MW (150 kDa)

Equation 2.4b. Theoretical maximum IAC sulfonamide binding (%)

$= 100* C*D*10^{-3}/A*B*E$

A, B, and C are defined in Equation 2.4a

D = observed sulfonamide extraction rate (μg/mL)

E = sulfonamide MW (g/mol).

2.5.2. Sulfonamide IAC Capacity Determination

For SMT and STZ and their derivatives the capacity of a 0.4 mL IAC was determined by exposing an IAC to saturating conditions of a sulfonamide in a buffer of PBS + 1% BSA. Saturating conditions were defined as the moles of sulfonamide added to a column in 4 mL PBS + 1% BSA, that would be equivalent to at least twice the actual holding capacity of the IAC in SMT or STZ mole equivalents. The capacity of anti-SMT IACs and anti-STZ IAC were based on SMT and STZ, respectively. Columns were exposed to saturating conditions of a sulfonamide, then they were washed with 5 mL PBS,

followed by 5 mL water, and then sulfonamides were eluted with 4.0 mL 10 mM HCl. The IACs were regenerated with 10 mL of 10 mM HCl. The BM assay was performed on the IAC eluants to determine concentrations of SMT, STZ and their N^4-acyl derivatives (NA-sulfonamide, NP-sulfonamide, and succinyl-sulfonamide). Instead of the BM Assay, NAHis-sulfonamide concentration was determined colorimetrically using the molar extinction coefficient of 22460 (Garden and Sporns, 1994). Duplicate 1.0 mL aliquots of the IAC eluant containing NAHis-sulfonamide were mixed with 0.5 mL 2 N NaOH, and then the absorbance measured at 436 nm, and the concentration determined using Equations 2.5 a, b, or c.

Equation 2.5a. [NAHis-sulfonamide] mole/L = 22460 (A_{436})(1cm)

Equation 2.5b. IAC capacity μg sulfonamide/ mL IAC = A*B/C

A = sulfonamide concentration (μg/mL) in the eluant determined by the BM assay

B = volume of eluant (4 mL)

C = volume of IAC (0.4 mL)

Equation 2.5c. IAC capacity (nmol sulfonamide/mL IAC)

= Equation 2.5a*10^3/MW sulfonamide

2.5.3. Competitive IAC Binding Capacity: Calculation of Inhibition and Cross-reactivity

A competitive binding experiment using 4 different IACs, made with immunopurified antibodies from sera 1A, 1B, 1C, or 1D, was as conducted such that both the reference sulfonamide and the competing derivatives were at saturating conditions. The sulfonamides were prepared as described in section 2.5.2. Equimolar mixtures of reference sulfonamide and their respective competing derivatives were made such that each compound was at the standard saturating level relative to the column capacity for the reference sulfonamide, SMT or STZ. The mixtures were applied to the IAC, eluted with 10 mM HCl, and then the SMT or STZ was quantified by the BM assay. SMT and its derivatives were added to anti-SMT IACs 1A and 1B, whereas STZ and its derivatives

were added to anti-STZ IACs 1C and 1D. No hydrolysis reaction of the samples prior to the BM assay was performed, so the derivatives were not measured (note: sulfonamide derivative controls analyzed by the BM assay were negative). The percent inhibition of the reference sulfonamide by the competitor derivative was determined by Equation 2.6.

Equation 2.6. Percent inhibition = 100*(A-B)/A

A = the maximum capacity of the IAC for the parent sulfonamide, SMT or STZ, when exposed to the IAC in saturating conditions

B = capacity of the column for the parent sulfonamide, SMT or STZ (68.7 $\pm$ 1.78 and 18.9 $\pm$ 3.9 nmol SMT for IACs 1A and 1B, respectively, and 70.5 $\pm$ 1.6 and 38.2 $\pm$ 0.4 nmol STZ for IACs 1C and 1D; note: the IAC volumes were variable and not necessarily equal, but the same IAC for each antibody source was used throughout the experiment).

The cross-reactivity of sulfonamide derivative competing with the parent sulfonamide for IAC binding, both at saturating conditions and equimolar concentrations, is given by Equation 2.7.

Equation 2.7. IAC cross-reactivity for a sulfonamide competitor = 100* A/(100-A)

A = Percent inhibition caused by a sulfonamide competitor as described by Equation 2.6.

2.5.4. IAC Bleed Rate

The IAC antibody bleed rate was determined using ELISA Format 1, and was based on the direct relationship between the antibody concentration and ELISA response. A standard antibody dilution was established at limiting concentration (1/60k), so that the antibody concentration tested would be directly proportional to the A_{450}. Antibody 1D was shown to have about 1.4 mg specific antibody/mL serum assuming it was pure IgG. This same antibody was immunopurifed, covalently attached to CNBr-activated Sepharose IAC, and tested for antibody bleed. The IAC washings were diluted and the antibody concentration was determined from its ELISA response relative to that of the control antibody (antibody 1D was 23 ng/mL at 1/60k). The IAC bleed rate was determined by Equation 2.8.

Equation 2.8. Antibody bleed rate from IAC washings = (A-B)*C*D/(E-B)

A = average ELISA A_{450} of diluted washing; B = average ELISA A_{450} of PBST blank

C = concentration of standard 1D antibody (23 ng/mL)

D = dilution factor of IAC washing

E = average ELISA A_{450} of positive control antibody

2.6. BRATTON-MARSHALL COLORIMETRIC ASSAY

The Bratton-Marshall (BM) assay is based on reaction of N-1-napthylethylenediamine dihydrochloride (NED) with a diazonium ion derivative of the N^4-amino group of a sulfonamide (Marshall Jr. and Babbitt, 1938). For testing the larger elution volumes from IAC capacity tests, duplicate 1.0 mL volumes of IAC eluants, 10 mM HCl blank solutions, or 10 µM sulfonamide standards in 10 mM HCl were mixed with 0.5 mL 3.5 N HCl. Next, 40 µL of 0.8% (w/v) sodium nitrite was added to each test tube, and the contents were mixed. After 2 min, 40 µL of ammonium sulfamate was added to quench excess sodium nitrite, mixed, and then 40 µL of 0.8% (w/v) NED was added and mixed. The pink colour was developed for 20 min in the dark before reading the absorbance at 545 nm.

For testing small volumes concentrated from micro-SPE columns, this method was miniaturized into 96-well, flat-bottomed microtiter plates (Costar, Corning Inc., Corning, NY). To each 100 µL of sample, buffer blank, or 10 µM sulfonamide standard in buffer, 100 µL of 3.5 N HCl was added. Next, 10 µL of 0.8% (w/v) sodium nitrite was added, and the plate was gently tapped to mix the contents. After 2 min, 10 µL of ammonium sulfamate was added, tapped again, and then 10 µL of NED was added. After developing colour for 20 min in the dark, the absorbance was read at 545 nm by a SpectraMax 190 microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA). Sulfonamide concentration was determined by Equation 2.9.

Equation 2.9. [Sulfonamide] µM = (A-B)*C*E/(D-B)

A = absorbance of sample

B = absorbance of blank

C = concentration of the sulfonamide standard (10 µM)

D = absorbance of standard

E = dilution factor of sample (usually 1.0)

When N^4-acyl derivatives (NA-sulfonamide, NP-sulfonamide, or Succinyl-sulfonamide), or succinyl-linked sulfonamide-protein conjugates were measured by this method, the N^4-acyl linkage first had to be hydrolyzed in basic conditions to free the primary aromatic amine necessary for diazonium ion formation. Hydrolysis was achieved by placing the aqueous sample in a sealed glass vial with 2 N NaOH in a 5:2 volume ratio, and then placing it in a boiling water bath for 30 min. Pure standards of the appropriate N^4-acyl sulfonamides were hydrolyzed in parallel and tested in the BM assay against the sample.

2.7. ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA) FORMATS

2.7.1. Standard Protocol and Equipment Common to all ELISA Formats

In all ELISA procedures, 96 well flat-bottomed plates (Costar 9017, Corning, NY) were used, and absorbencies were determined using a SpectraMax 190 microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA). Unless otherwise stated, all ELISA formats imply plates are coated with STZ-azo-BSA conjugate for testing antibodies from rabbits immunized with STZ-LPH, or plates were coated with SMT-azo-BSA conjugate for testing antibodies from rabbits immunized with SMT-LPH. The standard sulfonamide-BSA coating concentration was determined by using a conventional two dimensional checkerboard dilution, where coating antigen dilutions are tested against antibody dilutions in all possible combinations (Harlow and Lane, 1988). Next, to maximize ELISA sensitivity, sulfonamide-BSA coating rates and corresponding antibody dilutions were chosen from the checkerboard evaluation. The criterion to choose the coating antigen concentration was based on achieving a minimum adequate absorbance (A_{450}=1.5) for the set of antibodies that would guarantee a limited antibody state. In other words, it was required that the antibodies not be saturating the available antigen sites coated on the solid phase, but rather be approximately 50% or less of maximum saturation.

Unless otherwise stated, sulfonamide-azo-BSA conjugates were used to coat the plates at 60 ng /mL PBS, and then incubated overnight at 4°C. Next, plates were washed three times with 250 µL/ well of PBST. The type and concentration of anti-sulfonamide antibody with or without addition of competitive sulfonamides is unique for each ELISA format, and is described in detail for each one. Common to all ELISA formats, antibodies were diluted in PBST + 1% BSA, added to plates in 100 µL duplicates for a given treatment, incubated for 2 hr at 37 °C, and then washed again three times with PBST before addition of second antibody. All ELISA procedures had common second antibody and substrate stages. The second antibody was goat anti-rabbit immunoglobin G (heavy & light chains)-horseradish peroxidase conjugate (GAR-HRP), and was diluted to 1/3000 in PBST + 1% BSA. GAR-HRP was added to each well (100 µL /well) and incubated for 1 hr at 37 °C. The plates were then washed three times with PBST, and then 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well. After 40 min the enzyme-substrate reaction was stopped with the addition of 30 µL 2N H_2SO_4 and the absorbance was read at 450nm.

2.7.2. ELISA Format 1: Indirect ELISA for Determining Antibody Titre

This competitive ELISA format was used to determine the antibody titres of rabbit polyclonal sera to sulfonamides (see appendices, Chapter 9, Table 9.3, for titres), or to measure the amount of antibody that became detached, or "bled", from a CNBr-activated Sepharose column by the mass action of the application of large volumes of aqueous buffers (see appendices, Chapter 9, Table 9.8 for results). Standard protocol was followed for antigen coating, first and second antibody additions, substrate addition, and reading the plates. A solution's anti-sulfonamide activity could be determined by comparing PBST dilutions of the solution in question using the standard ELISA protocol. The titre of a serum or antibody solution was defined as the dilution required to achieve an A_{450} of 1.5. To determine what dilution constituted the titre, the antibody solution was serially diluted in duplicate in the ELISA plate itself using a multi-channel pipetor. Depending on the antibody and its titre, the range of dilutions tested would vary, typically from 1/1000 to 1/1,000,000. ELISA response was determined by subtracting the average

A_{450} of replicates PBST blanks from the average sample A_{450}. For standard procedures the average A_{450} of PBST blanks never exceeded 0.15.

To test for activity of a purified antibody relative to its serum, it was first diluted based on its original unpurified serum-volume equivalent. The titre of this diluted sample could then be determined and compared relative to the original unpurified serum. If the purified antibody had less activity than its corresponding serum after application of this serum-volume adjustment, this meant that sulfonamide-specific antibody activity in the purified fraction had diminished (either by antibody denaturation/inactivation or by loss of antibodies in the process).

2.7.3. ELISA Format 2 : Indirect ELISA to Evaluate Non-specific Binding against LPH and BSA

This competitive ELISA format was used to determine the non-specific binding of antibodies to BSA and LPH proteins, and the effect of immunopurification of antisera on this non-specific binding (see appendices, Chapter 9, section 9.2.3). Standard protocol was followed for first and second antibody additions, substrate addition, and reading the plates. Sulfonamide-conjugates and proteins were coated at 100 ng/mL instead of 60 ng/mL to accentuate the non-specific binding or specific cross-reactivity to the non-conjugated proteins. Immunopurified antibodies were diluted to 100 ng/mL, concentrations capable of binding the sulfonamide-protein conjugates approximately equivalently as the corresponding sera dilutions of 1/120,000. As in ELISA Format 1, immunopurified antibody activity was compared to corresponding serum activity based on a serum equivalence adjustment factor, so approximately the same amount of sulfonamide-specific antibody was compared in ELISA wells testing either the immunopurifed antibody or the serum. Antibody dilutions used to bind coating antigens were not saturating.

2.7.4. ELISA Format 3 : Indirect Competitive ELISA for Evaluation of a Linker-Arm Effect by Varying the Type of Competitor Ligand Derivative

ELISA Experiments 1 and 3 employed ELISA Format 3. The results are presented in sections 3.3.1 and 3.3.3. IC_{50} values for parent sulfonamides, sulfonamide analogues, and sulfonamide derivatives were determined using this indirect competitive

ELISA (Format 3). This format was the basis of the comparison of relative antibody affinity for these compounds, and ultimately in quantitatively describing the linker arm effect and also the importance of structural features of the N^1 substituent of sulfonamide molecule. The IC_{50} is inversely proportional to the affinity of the antibody to the ligand. This 50% inhibition point is the most responsive portion of the competitive assay function, and thus is the most sensitive comparative measure of antibody-ligand affinity for a set of related ligands (see appendix for competitive ELISA inhibition functions).

Ten sera were used from both the N^4-azo and N^4-succinyl immunization systems to determine if the linker arm type used in immunizations caused any differences in the relative IC_{50} values for sulfonamide derivatives. Cross-reactivity in competitive ELISA describes the relative affinity of an antibody for a hapten competitor compared to a reference compound, in this case, the parent sulfonamide, SMT or STZ. Greater than 100% cross relative reactivity therefore indicates a higher antibody affinity for the competitor ligand than for the parent sulfonamide.

Standard protocol was followed for antigen coating, first and second antibody additions, substrate addition, and reading the plates. After the plates were coated overnight and washed, the competitor haptens were diluted in PBST + 1% BSA and added to the plate along with PBST + 1% BSA blanks in 100 µL duplicates. Sulfonamide competitors were made in PBST +1% BSA from 10,000 µg/L DMSO stock solutions. Where serial dilutions of competitor haptens were required, the haptens with the highest concentrations were placed in the plates and serially diluted using a multi-channel pipetor. To the hapten solutions already in the plates, immunopurified antibodies or sera were added, diluted to their titres to give a B_o (PBST + 1% BSA blank) absorbance at 450 nm of approximately 1.5. (see appendices, Chapter 9, Table 9.3 for titre values) ("B" and "B_o" are defined as the absorbance of a sample and the PBST blank, respectively). B/B_o values for hapten competitions were determined by comparing a competition ELISA response (B) to the blank ELISA response (B_o; using PBST + 1% BSA) to give a B/B_o ratios. ELISA IC_{50} was determined for each sulfonamide dilution set as a relative measure of antibody affinity for that ligand. ELISA IC_{50} was defined as the concentration of competing free sulfonamide required to inhibit by 50% a limiting amount of antibody from binding the solid-phase sulfonamide-BSA antigen bound to the

plate. Antibody cross-reactivity to a related ligand was then defined as a function of IC_{50} by Equation 2.10.

Equation 2.10. Percent antibody cross-reactivity = 100*A/B

A = IC_{50} reference sulfonamide (usually the parent sulfonamide, SMT or STZ)

B = IC_{50} sulfonamide competitor.

The IC_{50} is a measurement that describes the sensitivity of the assay. It is influenced by antibody affinity and concentration. The IC_{50} measurement tends to be lower for antibodies with higher affinities, because higher affinity antibodies have higher titres, and therefore are used at lower concentrations to achieve the same standard ELISA response (1.5 at A_{450}). At lower antibody concentrations, each single molecule of competitor ligand has more influence in competing with each antibody molecule. Since these higher affinity antibodies are diluted more in a competitive ELISA, they tend to detect lower concentrations of the competitor sulfonamide being tested (i.e. have lower detection limits).

2.7.5. ELISA Format 4 : Indirect Competitive ELISA for Evaluation of a Linker-Arm Effect by Varying the Type of Coating Antigen

ELISA Experiment 2 (section 3.3.2 of Chapter 3) employed Format 4 to measure the linker arm effect of antibodies made from sulfonamides immunogens with azo or succinyl linking arms. The protocol used differences in the bound sulfonamide-BSA conjugate linking arms to measure the linking arm effect, instead of changes in the linking arm-type attachments of sulfonamide derivatives, as in ELISA Format 3.

Standard protocol was followed for antigen coating, first and second antibody additions, substrate addition, and reading the plates. Both ELISA plates were coated with either sulfonamide-succinyl-BSA or sulfonamide-azo-BSA, for both STZ and SMT sulfonamide conjugate types. For a given antigen coating, antibodies were compared based on the type of linker-arm used in the immunogens during immunization. Coating antigen and antibody concentrations were determined based on a two-dimensional checkerboard experiment to achieve both limiting antibody concentrations condition and an absorbance at 450 nm between 1.0 and 1.5 for a given coating antigen. The antigen

coating rates and the corresponding antibody dilutions are detailed in Table 2.2. The same antigen conditions were selected for comparing antibodies derived from immunizations using immunogens with different linker-arms. Therefore, the relative ability of the antibody to bind coating antigen under identical competition conditions would be a measure of linker-arm effect. Based on the above criteria antigen coating concentrations were 500 ng/mL and 250 ng/mL in PBS, 100 µL/well, for azo-linked and succinyl-linked BSA conjugates, respectively (for both STZ and SMT sulfonamide types). Immunopurified antibodies were applied to these antigens to achieve approximately equivalent absorbencies of in between 1.0 to 1.5 for the PBST blanks. The competitions with STZ or SMT and the determination of the subsequent IC_{50} values were described in ELISA Format 3. The relative IC_{50} quotient was determined for each of the anti-SMT and anti-STZ antibody specificity types by Equation 2.11.

Equation 2.11. =Acyl/Azo immunogen IC_{50} quotient = A/B

A = IC_{50} of antibody associated with the sulfonamide-succinyl-LPH immunogen and B = IC_{50} of antibody associated with the sulfonamide-azo-LPH immunogen.

Table 2. 2. Antibody dilutions[1] used for antigen coating types in ELISA format 4

Rabbit source for immunopurified antibody	2A Anti-azo-SMT	3B Anti-succinyl-SMT	2C Anti-azo-STZ	3A Anti-succinyl-STZ
Antibody dilution against 500 ng/mL sulfonamide-azo-BSA coating	1/80k	1/35k	1/40k	1/60k
Antibody dilution against 250 ng/mL sulfonamide-succinyl-BSA coating	1/60k	1/60k	1/40k	1/60k

[1]Antibody dilutions were in serum equivalents based on the volume of serum from which they were purified.

2.7.6. ELISA Format 5: Competitive Indirect ELISA for Quantification of Sulfonamide in Determining the Antibody Bleed Rate from Immunoaffinity Columns

Standard protocol was followed for antigen coating, first and second antibody additions, substrate addition, and reading the plates. This format followed the competitive protocol of Format 3, so that a sample with an unknown sulfonamide concentration could be quantified by interpolating its competitive ELISA response in relation to a known set of sulfonamide standards. The standards were diluted serially in PBST + 1% BSA from 0.3 nM to 3 μM. The coating and antibody conditions were SMT-azo-BSA (60 ng/mL) and antiserum 1B (1/120,000 dilution), and STZ-azo-BSA (60 ng/mL) and antiserum 1D (1/120,000), for SMT and STZ determinations, respectively. These assays served to quantify sulfonamides in the development of methodology for the SPIE extraction columns when the sensitivity of the BM assay was limiting.

2.8. SOLID PHASE IMMUNOEXTRACTION (SPIE) OF FORTIFIED ENVIRONMENTAL SAMPLES

2.8.1. Sampling and Storage of Environmental Samples

Two soil samples (3 kg) were collected from the top 20 cm of earth at two sites, one from a deciduous forest on the south bank of the North Saskatchewan River adjacent to the University of Alberta, Edmonton, Canada, approximately 15 metres higher than the river (soil 1), and the other at a cultivated agricultural field approximately 15 km south of Edmonton (soil 2) at a University of Alberta research facility at Ellerslie. Samples were mixed well, sealed in polyethylene bags, and stored at -20 °C until thawed for analysis. Composted manure was obtained from the Bioresource Engineering Group of the Department of Agricultural, Food and Nutritional Science at the University of Alberta, and was a mixture of dairy cow, poultry, and swine manures and beddings, containing approximately 40% carbon and 2.5% nitrogen by dry weight. There was no history of sulfonamides used in the feed rations of the animals which produced manure resources in this study (personal communications). Moisture contents of the soil and manure samples were determined by the average of differential weighing of duplicate 10 gram samples before and after heating for 20 h at 80°C in a forced air oven.

2.8.2. Pre-SPIE Sample Processing

Water (1000 mL), acidified with 5.0 mL of 1.0 M acetic acid adjusted to pH 5.0, was added to a glass 1.2 L narrow mouth reagent bottle. Zero or 100 g of soil or composted manure sample was added by funnel to each glass container. SMT and NA-SMT (3.60 nmol each) were added to each sample by adding 50.0 µL of a 72.0 µM SMT stock solution in DMSO and 53.7 µL of a 67.0 µM stock solution of NA-SMT in DMSO. These fortified samples were equivalent to 1.00 ng/L SMT and 1.15 ng/L NA-SMT based on 1 L of water added. SMT and NA-SMT (0.36 nmol each) were added to 1 L water samples (equivalent to 0.100 ng/L SMT and 0.115 ng/L, respectively) by adding 5.0 µL of a 72.0 µM SMT stock solution in DMSO and 5.4 µL of a 67.0 µM stock solution of NA-SMT in DMSO. DMSO (100.0 µL) was added to all unfortified controls. Samples were stirred on stirring plates for at least 1 hr at room temperature before being allowed to settle for 5 min. The liquid suspension from each sample was then decanted into centrifuge bottles and centrifuged at 5,000 x *g* for 20 min. Supernatants were dispensed into 1.2 L clean glass narrow mouth reagent bottles ready for concentration and purification procedures.

2.8.3. Preparation of Solid Phase Immuno-extraction (SPIE) Column System

The SPIE system used to purify and concentrate the SMT sulfonamides was comprised of three solid phase extraction steps used in tandem as follows: a primary concentration and crude purification step using HLB Plus cartridges, a secondary anti-SMT IAC chromatography step for further purification, and a final micro-SPE column concentration step. Oasis HLB Plus™ cartridges obtained from Waters Corp (Milford, MA) contained 225 mg of solid phase material and were used as is. SMT-specific immunoaffinity column were prepared as described in section 2.4.3. Micro-solid phase extraction (micro-SPE) columns were fashioned out of 200 µL bevelled pipet tips. The tips were plugged with sand, and then approximately 5 mg of Oasis HLB Plus packing material was added.

HLB Plus Solid Phase Extraction (SPE) Cartridges

Cartridges were preconditioned with 5 mL of ethanol followed by 10 mL of water. Cartridges were fitted into centrally-bored holes in #7 rubber stoppers, two-way

stopcocks were fitted to the other end of the cartridge, and these assemblies were mounted onto 1 L vacuum flasks. Sample supernatants were connected by flexible tubing (Tygon, Akron, OH) to the HLB Plus cartridges via stopcocks. Samples were drawn through cartridges by manifold vacuum filtration at 20 – 30 mL/min, and the stopcocks were adjusted to ensure approximate flow rate equivalency among samples. The cartridges were washed with 10 mL water, the excess water removed from the cartridges by vacuum, then sulfonamides were eluted with 3 mL ethanol.

Anti-SMT Immunoaffinity Column (IAC)

Immunopurifed antibodies from serum 1B were used for this IAC system. Ethanol eluant (3 mL) from the HLB Plus cartridge was diluted by adding it to 12 mL of PBS. This diluted sample was then loaded by vacuum at 1 mL/min to a 0.4 mL anti-SMT IAC column. IAC columns were washed with 5 mL of PBS, sulfonamides were eluted with 4 mL of 10 mM HCl, and the acidic eluants were collected into test tubes containing 3.0 µL of 0.379 mM SMR in DMSO. IAC columns were regenerated with 10 mL of 10 mM HCl followed by 10 mL of PBS. An aliquot of the final 4 mL of acidic eluant used for column regeneration was collected and analyzed in the same manner as the samples to verify the removal of all sulfonamides from the column.

Micro-SPE Column

Micro-SPE columns were inserted into a filtration manifold made from a #14 rubber stopper bored with twelve 4 mm holes inserted into a 400 mL beaker and connected to a vacuum. Pipet tips (1000 µL) were inserted into the top of the micro-SPE columns to provide a holding reservoir for solvent and sample addition, and then the columns were conditioned with 1 mL of ethanol followed by 1 mL of water. The acidic eluants, containing SMR as positive control and recovery standard, were applied to the pre-conditioned micro-SPE columns and rapidly drawn through by vacuum. The micro-HLB columns were washed with 1 mL of water and detached from the rubber vacuum manifold after they were dry. Ethanol (50 µL) was added to each column and forced through with a positive displacement pipetor, and the eluant was collected in a glass vial. Ethanol eluants were evaporated to dryness with a stream of nitrogen gas. Sulfonamide residues were dissolved in 10 µL DHB matrix (10 mg/mL in 50% ethanol).

2.9. SOLID PHASE IMMUNOEXTRACTION OF PIG FARM SAMPLES

2.9.1. Sampling and Storage of Farm Samples

Several environmental samples were obtained in July of 2003 from a local pig farm within 200 km of Edmonton. This farmer had farmed since 1987, and had records of using SMT in his pig feed for the 23 years previous to the collection of these samples. The farmer had 120 sows at the time, which he operated from farrow-to-finish. To avoid cross contamination, samples were collected in sequence starting with those expected to have the least amount of sulfonamides (field samples) and ending with samples expected to have the highest (lagoon 2, lagoon 1, weaner barn, then feed, in that order). Manure samples from surface of the pit under the weaner pigs in the weaner barn were collected directly into 1 L polyethylene tubs and secured with lids. Waste material from lagoons 1 and 2 were obtained from a depth of approximately 2 m. A 1 L polypropylene bottle was attached to the end of a 3 m pole, the bottle was plugged with a cork, and opened for sampling with a line connected to the stopper. Several water samples from two different ponds in a cut-line pasture and one from the farm's well were collected using 1 L plastic polypropylene bottles. Soil samples were obtained from two different sites at the pig farm. Site 1 was the cut-line pasture that had the two ponds and where waste from lagoon 1 was commonly spread. Site 2 was an agricultural field using for growing grain, and waste material from lagoon 2 was commonly spread there. Several 1 kg soil samples were obtained from each site by digging with a shovel to a depth of about 20 cm, and then placed into polyethylene bags and sealed. The water, manure, lagoon, and soil samples were stored at 4 °C until analysis within two weeks.

2.9.2. Pre-SPIE Sample Processing of Farm samples

Water Samples

Water samples (1000 mL) were acidified with 5.0 mL of 1.0 M acetic acid adjusted to pH 5.0, and then added to a glass 1.2 L narrow-mouth reagent bottle. NP-SMT (30.0 µL of a 360 µM stock in DMSO) was added as internal standard to each water sample and stirred for 5 min with a magnetic stir bar.

Soil Samples

Soil samples were treated with solvents based on a modified procedure described by Pfeifer et al (2002). Soils (200 g) were placed into 1 L centrifuge bottles, and then water (200 mL), NaCl (40 g), 1 M sodium acetate (pH 5, 2 mL), and NP-SMT (30.0 µL of a 360 µM stock in DMSO) were added to each sample. The buffered soil mixtures were each treated with 100 mL of n-hexane and ethyl acetate (95:5, v/v) for lipid removal. Mixtures were shaken for 10 min on a horizontal shaker at RT and 3 cycles/s. Phases were separated by centrifugation at 3400 G for 15 min and the organic phase was aspirated and discarded. Sulfonamides were extracted twice from the aqueous phase by two liquid-liquid extractions with 100 mL ethyl acetate, with shaking (10 min) and centrifugation. Ethyl acetate extracts for each sample were pooled and then evaporated by rotary evaporation. Sulfonamides were suspended in 400 µL of ethanol, and transferred quantitatively to a glass vial 3.0 mL PBS.

Manure Samples

Manure pit and lagoon waste samples were treated with solvents based on a modified procedure described by Pfeifer et al (2002). Samples were filtered through glass wool, then transferred (10.0, 1.0, and 0.1 mL for waste lagoon 2, waste lagoon 1, and weaner barn pit samples, respectively) to 50 mL centrifuge bottles, and made up to approximately 10 mL volume with water. NaCl (2 g), 1 M sodium acetate (pH 5, 2 mL), and NP-SMT (30.0 µL of a 360 µM stock in DMSO) as internal standard were added to each sample. The buffered manure mixtures were each treated with 10 mL of n-hexane and ethyl acetate (95:5, v/v) for lipid removal. Mixtures were shaken for 10 min on a horizontal shaker at RT and 3 cycles/s. Phases were separated by centrifugation at 3400 x g for 15 min and the organic phase was aspirated and discarded. Sulfonamides were extracted twice from the aqueous phase by two liquid-liquid extractions with 10 mL ethyl acetate, with shaking (10 min) and centrifugation. Ethyl acetate extracts for each sample were pooled, and then evaporated to dryness under vacuum with a rotary evaporation. Sulfonamides were dissolved in 400 µL of ethanol, and transferred quantitatively to a glass vial with 3.0 mL PBS.

Feed Sample

Feed samples (1.000 g) were weighed into beakers. Water (100 mL) and NP-SMT (400.0 µL of a 360 µM stock in DMSO) as internal standard were added, and then the samples were homogenized for 2 min at 10,000 rpm using a Polytron™ (Brinkmann, Westbury, NY) homogenizer. The homogenized samples were centrifuged at 3400 x g for 15 min, and 5.0 mL of the supernatant was transferred to 50 mL centrifuge bottles and made up to approximately 10 mL with water. NaCl (20 g) and 1 M sodium acetate (pH 5, 2 mL) were added, and then the buffered feed samples were treated with n-hexane and ethyl acetate (95:5, v/v) for lipid removal as described for the manure samples. Ethyl acetate extracts were pooled, and then evaporated to dryness under vacuum with a rotary evaporation. Sulfonamides were dissolved in 400 µL of ethanol, and transferred quantitatively to a glass vial with 3.0 mL PBS.

2.9.3. Preparation of Solid Phase Immuno-Extraction (SPIE) Column System for Farm Samples

HLB Plus Cartridges

Cartridges were preconditioned with 5 mL of ethanol followed by 10 mL of water. The water samples (1000 mL) were applied to the cartridges as described for the solid phase immunoextraction of fortified samples in section 2.8.3. The soil, manure and feed samples were much smaller in volume (about 3 mL), and were applied to conditioned cartridges by 5 mL syringe over 2 min. Cartridges were washed with 10 mL water, dried by vacuum, and then eluted with 3 mL ethanol.

Anti-SMT IAC and Micro-SPE Columns

Immunoextraction of HLB Plus cartridge farm sample eluants and the subsequent micro-SPE concentration of sulfonamides was the same as described for the solid phase immunoextraction of fortified samples in section 2.8.3. SMR (10.0 µL of 360 µM stock in ethanol) as internal standard and DHB (10 µL of 10 mg/mL in 50% ethanol) were added and mixed to sulfonamide residues in the microvials.

2.10. MATRIX ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS) SULFONAMIDE ANALYSIS

2.10.1. General MALDI-TOF MS Analysis Common to all Experiments

Ten-position target probes were made of highly polished stainless steel (Bruker Analytical Systems Inc., Billerica, MA). A matrix solution of 2,5-dihydroxybenzoic acid (DHB) was prepared at 10 mg/mL in 50% aqueous ethanol. Dried micro-SPE column eluants were dissolved with 10 µL of DHB solution, and approximately 1 µL was applied to each of 3 spots on the target probe. The matrix and sample were co-crystallized on the probe by allowing the solvent to evaporate under a fan in ambient conditions. MALDI-TOF MS spectra were acquired on a linear Bruker Proflex™ III instrument (Bruker Analytical Systems, Billerica, MA) equipped with a nitrogen laser (337 nm). This instrument was set in positive ion mode with an extraction potential of 20 kV and a delay of 6000 ns. All mass spectra were generated by collecting 100 laser shots. Laser strength and detector voltage were adjusted to obtain optimal signal-to-noise ratios and high resolution. Three spectra were generated for each sample and responses were averaged. Analyte response was defined as the sum of peak heights from proton, sodium, and potassium adducts together with any fragments $(M+H)^+$, $[M+Na]^+$, $[M+K]^+$, and $[M-SO_2+H]^+$ ions, where M = mass for the unfragmented analyte). Peaks were detected if they were three times greater than the general background noise in the spectral region of interest.

2.10.2. MALDI-TOF MS Analysis of Fortified Environmental Samples

Response factors of SMT and NA-SMT were determined relative to the internal standard SMR. SMT, NA-SMT, and SMR stock solutions in DMSO (3.60 mM, 3.35 mM, and 3.79 mM, respectively) were mixed (10.0 µL each), 3.0 µL of the mixture was added to 3.0 mL PBS. This solution was processed normally through a micro-SPE column and three MALDI-TOF MS spectra were generated. The molar response ratio for SMT or NA-SMT was determined based on an average of these three spectra according to Equation 2.12. Response ratios were 0.99 ± 0.12 and 0.42 ± 0.06 for SMT/SMR and NA-SMT/SMR, respectively (n=3).

Equation 2.12. Molar response ratio of analyte/SMR = A*D/B*C

A = sum of peak heights of SMT or NA-SMT

B = sum of peak heights of SMR

C = moles of SMT or NA-SMT analyte processed through micro-HLB column

D = moles of SMR internal standard processed through micro-SPE column

SPIE Recovery Rate Determination of Fortified SMT and NA-SMT with MALDI-TOF MS

Response ratios of SMT and NA-SMT relative to SMR were used to estimate their recoveries from the first two steps of SPIE, and the Bratton-Marshall assay was used to estimate micro-SPE column efficiency. Percent recoveries of sulfonamide from SPIE were estimated by MALDI-TOF MS using Equation 2.13.

Equation 2.13. Percent recovery of analyte from SPIE = 100 x A*D*F/B*C*E

A = sum of peak heights of SMT or NA-SMT

B = sum of peak heights of the internal standard, SMR

C = response ratio determined by Equation 2.12 for the analytes SMT or NA-SMT

D = moles of internal standard (SMR) added to system (1.14 nmol)

E = moles of SMT or NA-SMT added originally to sample (3.60 nmol)

F= average colorimetric determination of sulfonamide (SMT, NA-SMT, and SMR) fractional recovery for micro-SPE column ($F=0.81 \pm 0.01$; n=3). Factor F is necessary to compensate for loss at the micro-SPE column step, because the internal standard was added just before this last step and so only estimates the recovery of the first two stages of the SPIE system. All errors reported in this paper are the standard error of the mean.

2.10.3. MALDI-TOF MS Analysis of Pig Farm Samples

NP-SMT was added to samples as a quantification internal standard at the beginning prior to sample purification and concentration, and then SMR was added at the end of the clean-up process as a positive control and a means to determine NP-SMT recovery rate.

Molar Response Ratio Determination for Quantification with MALDI-TOF MS in Farm Samples

Response factors of SMT and NA-SMT were determined relative to the internal standard, NP-SMT. Sulfonamide stock solutions (3.00 mM) in ethanol were made from 1000 µg/L sulfonamide DMSO stock solutions. SMT or NA-SMT were added in duplicate to glass microvials (9.0, 18.0, 36.0, 72.0, 126.0, and 180.0 µL volumes of the 3.00 mM stock solutions) together with 36.0 µL of 3.00 mM NP-SMT stock solution. The sulfonamides were evaporated to dryness at 100 °C, then allowed to cool to RT. DHB matrix solution was added (300 µL of 10 mg/mL DHB in 50% ethanol) and the vials were mixed well. The mixtures were applied to a target probe, representing approximately 0.36 nmol of NP-SMT per spot and a range of 0.090 to 1.80 nmol of SMT or NA-SMT per spot. Three mass spectra were generated for each sulfonamide mixture. Molar response ratios for SMT or NA-SMT were determined based on an average of these spectra according to Equation 2.14.

Equation 2. 14. Molar response ratio of analyte/NP-SMT = A*D/B*C

A = sum of peak heights of SMT or NA-SMT analyte

B = sum of peak heights of NP-SMT, the internal standard

C = moles of SMT or NA-SMT analyte on the target spot

D = moles of NP-SMT internal standard on the target spot

Response ratios were 2.46 and 0.93 for SMT/NP-SMT and NA-SMT/NP-SMT, respectively (n=3), based on the linear correlations of their relative responses with their molar ratios (R^2 = 0.996 and 0.992, respectively).

Quantification of SMT and NA-SMT relative to NP-SMT in Farm Samples with MALDI-TOF MS

Analyte responses were determined relative to the internal standard, NP-SMT. For quantification purposes SMT, NA-SMT and NP-SMT were assumed to be recovered equally in the liquid/liquid extraction and SPIE processes. Although recovery experiments for SMT and NA-SMT from fortified soil and manure matrices had not been performed using the extraction procedure of section 2.9.3, this equivalent recovery

assumption was reasonable based on reported sulfonamide extraction rates with n-hexane and ethyl acetate (Pfeifer et al., 2002) and IAC recoveries reported in Chapter 4. Future recovery experiments of all three sulfonamides are required to validate this quantification method. The concentrations of SMT and NA-SMT in farm samples were determined using Equation 2.15. Each farm sample was processed once as described in section 2.9.3, and the extracted sulfonamides were analyzed in triplicate by MALDI-TOF MS.

Equation 2.15. Concentration of SMT or NA-SMT (nM) = A*C*E/B*D

A = sum of peak heights of SMT or NA-SMT analyte

B = sum of peak heights of NP-SMT, the internal standard

C = molar response ratio of SMT or NA-SMT analyte relative to NP-SMT (Equation 2.14)

D = volume (L) or mass (kg) of sample analyzed

E = nmol of NP-SMT fortified into the sample (e.g. 3.00×10^{-5} L * 360 μM = 10.8 nmol for the water, manure, and soil samples)

Molar Response Ratio Determination of SMT, NA-SMT, and NP-SMT Relative to SMR for Recovery Determination in Farm Samples Using MALDI-TOF MS

Response factors of SMT, NA-SMT, and NP-SMT relative to the internal standard SMR were determined as follows: SMT, NA-SMT, NP-SMT, and SMR were added to microvials (10, 20, 20, and 8 μL of 360 μL M ethanolic stock solutions, respectively). They were dried at 100 °C, dissolved with 10 μL of DHB matrix solution, and approximately 1 μL was applied to each of 3 spots on the target probe. The molar response ratio of SMT, NA-SMT, and NP-SMT relative to SMR was determined from an average of 3 replicate spectra according to Equation 2.16. Response ratios were 1.15 ± 0.08, 0.73 ± 0.06, 0.77 ± 0.05 for SMT/SMR, NA-SMT/SMR, and NP-SMT/SMR, respectively (n=3).

Equation 2.16. Molar response ratio of analyte/SMR = A*D/B*C

A = sum of peak heights of SMT, NA-SMT, or NP-SMT analyte

B = sum of peak heights of SMR, the internal standard

C = moles of SMT, NA-SMT, NP-SMT analyte on the target spot

D = moles of NP-SMT internal standard on the target spot

Recovery of NP-SMT in Farm Samples Relative to SMR with MALDI-TOF MS

SMR (10.0 µL of 360 µM stock solution in ethanol) was pipetted into the microvials after sulfonamides were eluted from the micro-SPE step of the purification process described previously section 2.9.3. Solvent was evaporated at 100 °C, and sulfonamides were re-dissolved in 10 µL of DHB solution, and approximately 1 µL was applied to each of 3 spots on the target probe. Response ratios of NP-SMT relative to SMR were used to estimate recovery rates. Percent recoveries of NP-SMT for liquid-liquid extraction and/or SPIE for farm samples was estimated using Equation 2.17. Each farm sample was processed once, and the extracted sulfonamides were analyzed in triplicate by MALDI-TOF MS.

Equation 2.17. Percent recovery of NP-SMT fortified in the farm samples

= 100 x A*D/B*C*E

A = sum of peak heights of NP-SMT

B = sum of peak heights of SMR, the recovery standard

C = specific response ratio of NP-SMT relative to SMR, the recovery standard (0.77)

D = moles of internal standard SMR added to system (10.0 µL X 3.60 µM = 3.60 nmol)

E = moles of NP-SMT, added originally to sample (30.0 µL X 3.60 µM = 7.20 nmol)

Fortified SMT, NA-SMT, and NP-SMT Standards in a Water Recovery Experiment Relative to SMR with MALDI-TOF MS

A recovery experiment was performed to determine the recovery rates of equimolar concentrations of SMT, NA-SMT, and NP-SMT relative to SMR. SMT, NA-SMT, and NP-SMT (10, 20, and 20 µL of 360 µL M stock solutions in ethanol, respectively) were pipetted in combination into either 1 L water (buffered with 5.0 mL of 1.0 M sodium acetate, pH 5.0) or 4 mL PBS with 20% ethanol, for total SPIE or IAC/micro-SPE recovery rate determinations, respectively. SPIE and the IAC/micro-SPE procedures followed the protocols previously described. Immunopurifed antibodies from serum 1B were used for these IACs. SMR (8.0 µL of 360 µM stock solution in ethanol) was

pipetted into the microvials after sulfonamides were eluted from micro-SPE columns, and the solvents were evaporated at 100 °C. Sulfonamides were re-dissolved with 10 µL of DHB solution, and approximately 1 µL was applied to each of 3 spots on the target probe. Response ratios of SMT, NA-SMT, and NP-SMT relative to SMR were used to estimate their recovery rates. Percent recoveries of sulfonamide from SPIE were estimated by MALDI-TOF MS using Equation 2.18. Replicates were extracted by SPIE in triplicate, and then the mean recovery rate for each sample was determined.

Equation 2.18. Percent recovery of analyte from SPIE or IAC/micro-SPIE = 100 x A*D/B*C*E

A = sum of peak heights of SMT, NA-SMT, or NP-SMT

B = sum of peak heights of SMR, the recovery standard

C = specific response ratio of SMT, NA-SMT, or NP-SMT analyte relative to SMR internal standard (1.15, 0.73, and 0.77, respectively)

D = moles of internal standard SMR added to system (2.88 nmol)

E = moles of analyte, SMT, NA-SMT, or NP-SMT, added originally to sample (3.60, 7.20, and 7.20 nmol, respectively).

2.10.4. Simultaneous IAC Recovery Experiment of Several Related Sulfonamides using MALDI-TOF MS

Immunopurifed antibodies from serum 1B were used for this IAC system. A single IAC was challenged to simultaneously extract both sub-saturating and saturating masses of equimolar mixtures of SMT, NA-SMT, SMR, SPD, SDA, and STZ. A mixed stock solution was made containing all six sulfonamides. This mixed, equimolar sulfonamide stock solution was made by taking appropriate volumes of 10,000 µg/L sulfonamide stock solutions in DMSO and adding them to PBS + 1% BSA to give a final concentration of 350 µM for each sulfonamide. The mixed sulfonamide stock was added to 6 test tubes (10, 20, 50, 100, 300, and 1000 µL) and made up to 2.00 mL with PBS + 1% BSA and are denoted as trials a, b, c, d, e, and f respectively. Samples were loaded onto the IAC by gravity, washed with 5 mL PBS, and 5 mL water, and eluted with 4 mL 10 mM HCl into a test tube containing NP-SMT internal standard (10, 20, 50, 50, 50, and

50 µL of 350 µM stock for trials a, b, c, d, e, and f respectively), and mixed. Also, 10 µL of the 350 µM mixed sulfonamide stock and 10 µL of 350 µM NP-SMT were added to 4.0 mL 10 mM HCl and mixed, to be used for determination of MALDI-TOF MS sulfonamide response factors. The NP-SMT – fortified IAC eluants and the 10 mM HCl solution fortified with seven sulfonamides were processed by micro-SPE columns as described for the solid phase immunoextraction of fortified environmental samples.

Absolute and relative recoveries were determined for the six sulfonamides using MALDI-TOF MS. Response factors of SMT, NA-SMT, SMR, SPD, SDA, and STZ were determined relative to the internal standard, NP-SMT, as described in Equation 2.19. Response ratios were 1.95 ± 0.06, 0.97 ± 0.01, 1.14 ± 0.02, 0.74 ± 0.01, 0.54 ± 0.01, and 0.41 ± 0.06 for SMT, NA-SMT, SMR, SDA, SPD, and STZ, respectively ($\pm$ standard error of the mean; n=3).

Equation 2.19. Molar response ratio of analyte/NP-SMT = A*D/B*C

A = sum of peak heights of each specific sulfonamide analyte

B = sum of peak heights of NP-SMT, the internal standard

C = nmol of analyte in mixture

D = nmol of NP-SMT internal standard in mixture.

The percent absolute recovery and relative recoveries for each sulfonamide were determined by Equations 2.20 and 2.21, respectively. Triplicate spectra were generated for each trial and the mean recovery rate was determined.

Equation 2.20. Percent absolute recovery for a sulfonamide = 100 x A*D/B*C

A = sum of peak heights of a sulfonamide analyte

B = sum of peak heights of NP-SMT, the internal standard

C = specific response ratio of analyte in relation to NP-SMT (see Equation 2.19)

D = moles of internal standard NP-SMT added to mixture (3.5, 7.0, 17.5, 17.5, 17.5, and 17.5 nmol for trials a, b, c, d, e, and f respectively)

Equation 2.21. Relative recovery for a sulfonamide = A*D/B*C*E*F

A, B, C, D, and E are defined in Equation 2.20

F is the sum of absolute recoveries for each of the six sulfonamides (absolute recovery for each sulfonamide = A*D/B*C*E nmol).

2.11. STATISTICAL ANALYSIS

The statistical significance of data was reported at the 95 % confidence level, and determined using a general linear model procedure of the SAS program (S.A.S.Institute Inc., 1989). The Student-Newman-Keuls test was used to assign significant difference to treatments. Unless specifically stated otherwise, variation of means was reported as the standard error of the mean. Polynomial regression analyses were determined using Microsoft Excel 2002. Third order polynomial functions were used to approximate the competitive ELISA response curves measured as B/Bo as a function of competitor molar concentration (R^2 values all greater than 0.98) ("B" and "Bo" are defined as the absorbance of a sample and the PBST blank, respectively). The IC_{50}[1] for each replicate competitor serial dilution set was interpolated from a function by solving for B/Bo = 0.5, and then the sulfonamide cross-reactivity[2] could be calculated relative to the parent or reference sulfonamide. Competitor ligands were able to inhibit antibody binding (B) to near zero values (see appendices, Chapter 9), so this IC_{50} definition was considered a legitimate measurement of the specific interaction of the ligand with the antibody.

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[1] ELISA IC_{50} was defined as the concentration of competing free sulfonamide required to inhibit by 50% a limiting amount of antibody from binding the solid-phase sulfonamide-BSA antigen coated to the plate.

[2] Percent antibody cross-reactivity = 100*A/B

A = IC_{50} reference sulfonamide (usually the parent sulfonamide, SMT or STZ)

B = IC_{50} sulfonamide competitor.

Chapter 3

DEVELOPMENT AND CHARACTERIZATION OF SULFONAMIDE-SPECIFIC ANTIBODIES

3.1. INTRODUCTION

This chapter presents the development of rabbit anti-sulfonamide polyclonal antibodies, and then their characterization by competitive enzyme-linked immunosorbent assay (ELISA). The ELISA technique characterized the affinity and specificity of these antibodies to derivatives and analogues of sulfonamides, and served as the foundation of information used to later assess their potential utility in sulfonamide immunoextraction (Chapter 4) for sulfonamide residue analysis (Chapters 5 and 6).

3.2. DEVELOPMENT OF ANTIBODIES

Sulfonamides, sulfamethazine (SMT) or sulfathiazole (STZ), were covalently linked to *Limulus polyphemus* hemolymph (LPH) with an azo-linker arm or succinyl-linker arm, making 4 different immunogens used to immunize a total of 10 rabbits (Figure 3.1). Rabbits were immunized in three separate batches using SMT or STZ conjugated to (LPH) (Table 3.1). Batches 1 and 2 represent eight rabbits immunized with SMT or STZ linked to LPH by the diazo reaction. Batch 3 immunizations were performed using STZ-LPH and SMT-LPH conjugates linked by a N^4-succinyl linkage so that the resulting antibodies could be used to further evaluate the linker arm effect phenomenon described in the introduction (see Chapter 1, for the background on antibody-hapten and -linker arm interactions; sections 1.5.2 through 1.5.4).

Table 3.1. Batch designation and sulfonamide immunogen used for 10 rabbits

Batch	Sulfonamide immunogen[1]			
	SMT-azo-LPH	STZ-azo-LPH	SMT-succinyl-LPH	STZ-succinyl-LPH
Batch 1	1A, 1B	1C, 1D		
Batch 2	2A, 2B	2C, 2D		
Batch 3			3A	3B

[1]Sulfonamide-protein conjugates are described above with the following abbreviations: sulfamethazine = SMT; sulfathiazole = STZ; LPH = *Limulus polyphemus* hemolymph protein; azo = azo linkage; succinyl = succinyl linkage

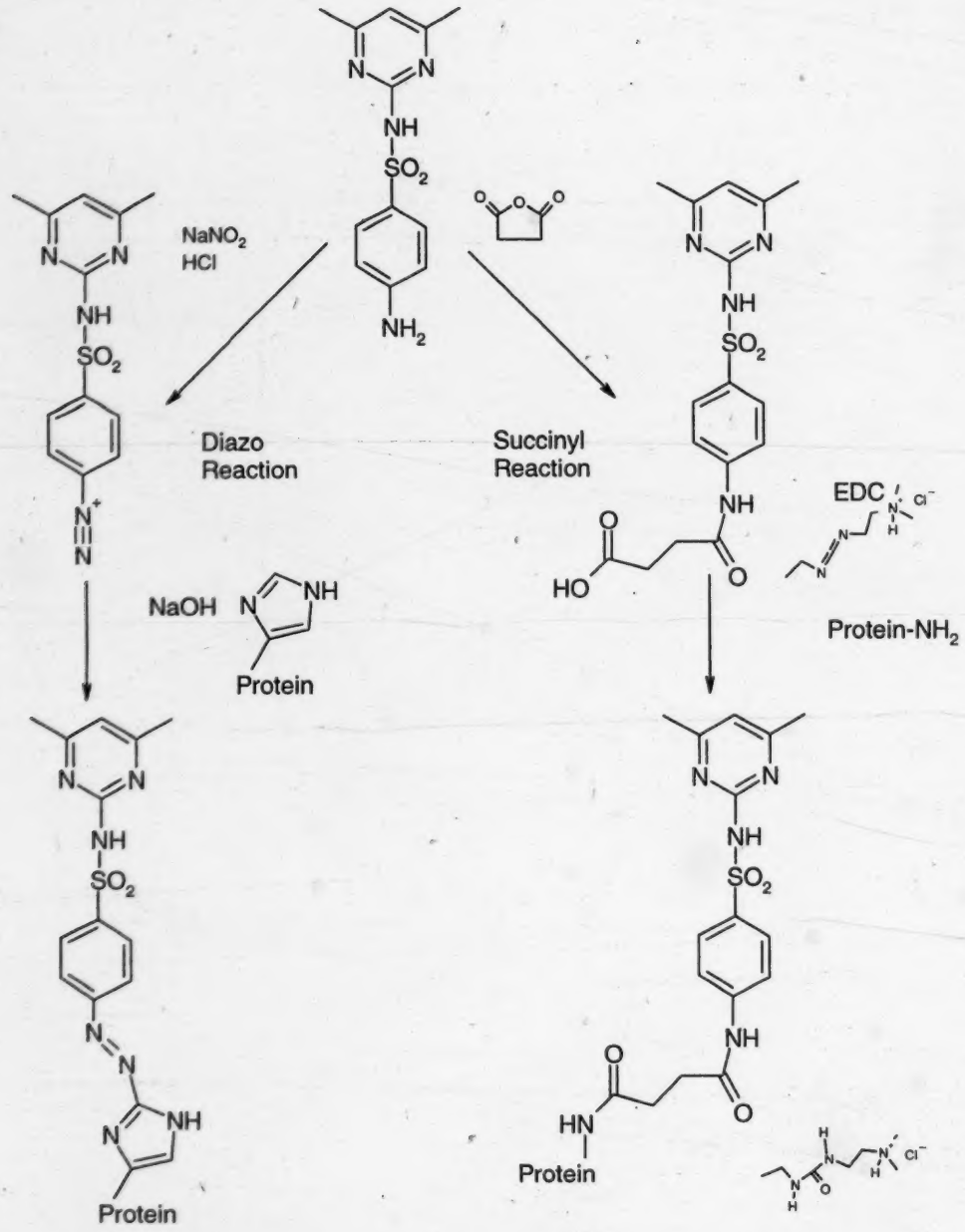

Figure 3.1. Preparation of azo- and succinyl-linked sulfamethazine-protein conjugates.

Molar incorporation rates of sulfonamide-protein conjugates used for immunization and in ELISA, the immunopurification of polyclonal antibodies, and the determination of antibody titres and yields are all presented in the appendices (Chapter 9). The immunopurification enrichment process is briefly described here. The sulfonamide-specific antibodies were isolated by first immobilizing an appropriate sulfonamide-bovine serum albumin (BSA) conjugate onto a CNBr-activated Sepharose 4B solid phase (STZ-azo-BSA conjugate for anti-STZ antibody purification and SMT-azo-BSA conjugate for anti-SMT antibody purification). These columns could then serve to isolate specific antibodies in sera since anti-sulfonamide antibodies could attach temporarily to an immobilized sulfonamide hapten, and then could be eluted.

Along with the corresponding antisera, these sulfonamide-specific antibodies were then evaluated extensively by ELISA as presented in this Chapter. Later, Chapter 4 describes the work to immobilize these specific antibodies onto the same type of CNBr-activated Sepharose 4B solid phase medium used to make the original antibody enrichment columns. These columns would subsequently serve to extract sulfonamide haptens, analogues, and derivatives from various samples.

3.3. ANTIBODY CHARACTERIZATION BY ELISA

3.3.1. ELISA Experiment 1: Evaluation of a Linker Arm Effect Using Indirect Competitive ELISA by Varying the Competitor Ligand Derivative

The antibody linker arm effect for the 10 antibodies was evaluated using competitive ELISA. The linker arm effect is a phenomenon regarding antibody specificity as it is dependent on how the hapten was linked to the protein that was used as immunogen. With respect to this immunogen linker arm, an antibody is expected to have a relatively lower affinity to the free hapten itself compared to either a hapten-protein conjugate that contains the same linker arm, or to hapten derivatives possessing these linker attachments. This linker arm effect was important to characterize because it has significant implications in making immunoaffinity columns (IACs) for subsequent hapten purification systems. One problem in working with hapten polyclonal antibodies is to subsequently immunopurify the hapten-specific antibody fraction (Ben Rejeb et al., 1998a; Choi et al., 1995; Delaunay et al., 2000). The reason this procedure is not

commonly reported is likely due to irreversible antibody binding of the hapten and associated linkages to the solid phase. Another more relevant reason linker arm effects are not desirable, is that the antibodies used in IAC may not have equivalent affinity to a hapten and its related derivatives, thus resulting in their unequal IAC recoveries.

A competitive ELISA (Format 3) was used to determine the presence and degree of a linker arm effect for all ten polyclonal antibodies manifested toward various sulfonamide derivatives of SMT and STZ (Figure 3.2). N^4-acyl derivatives (NA-sulfonamide, NP- sulfonamide, and succinyl-sulfonamide) were used to approximate the linker arm effect relative to parent sulfonamide in rabbits immunized with sulfonamides linked to LPH with an N^4-succinyl linkage (hereafter referred to as N^4-succinyl immunogen system). N^4-histidine and tyrosine amino acid-azo-sulfonamide derivatives (NAHis-sulfonamide and NCATyr-sulfonamide) were used to approximate the linker arm effect relative to parent sulfonamide in rabbits immunized with sulfonamides linked to LPH with an N^4-azo-linkage (hereafter referred to as the N^4-azo immunogen system).

Each antibody competition set was comprised of 10 duplicate ELISA competitions in individual microtiter plate wells. In a given competition, a constant amount of bound sulfonamide-BSA conjugate competed with a test sample of soluble sulfonamide (varied for 10 different concentrations representing a million fold dilution range), for a given soluble antibody (at a constant concentration). The ELISA responses of these competitions were compared to a "zero" soluble sulfonamide control (i.e. no competition control). Each competition set was differentiated by the source of antibody used and the type of soluble sulfonamide analogue or derivative competitor used. A remarkable variety was found in both the IC_{50} values[1] for parent sulfonamides for different antibodies, and also in the relative patterns of cross-reactivity[2] with the sulfonamide derivatives within each antibody competition. The complete competitive ELISA curve functions and the corresponding IC_{50} and cross-reactivity values represent

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[1] ELISA IC_{50} was determined for each sulfonamide dilution set as a relative measure of antibody affinity for that ligand. ELISA IC_{50} was defined as the concentration of competing free sulfonamide required to inhibit by 50% a limiting amount of antibody from binding the solid-phase sulfonamide-BSA antigen bound to the plate.

[2] Antibody cross-reactivity to a related ligand was then defined as a function of IC_{50}. Percent antibody cross-reactivity = 100*A/B; A = IC_{50} reference sulfonamide (usually the parent sulfonamide, SMT or STZ) and B = IC_{50} sulfonamide competitor.

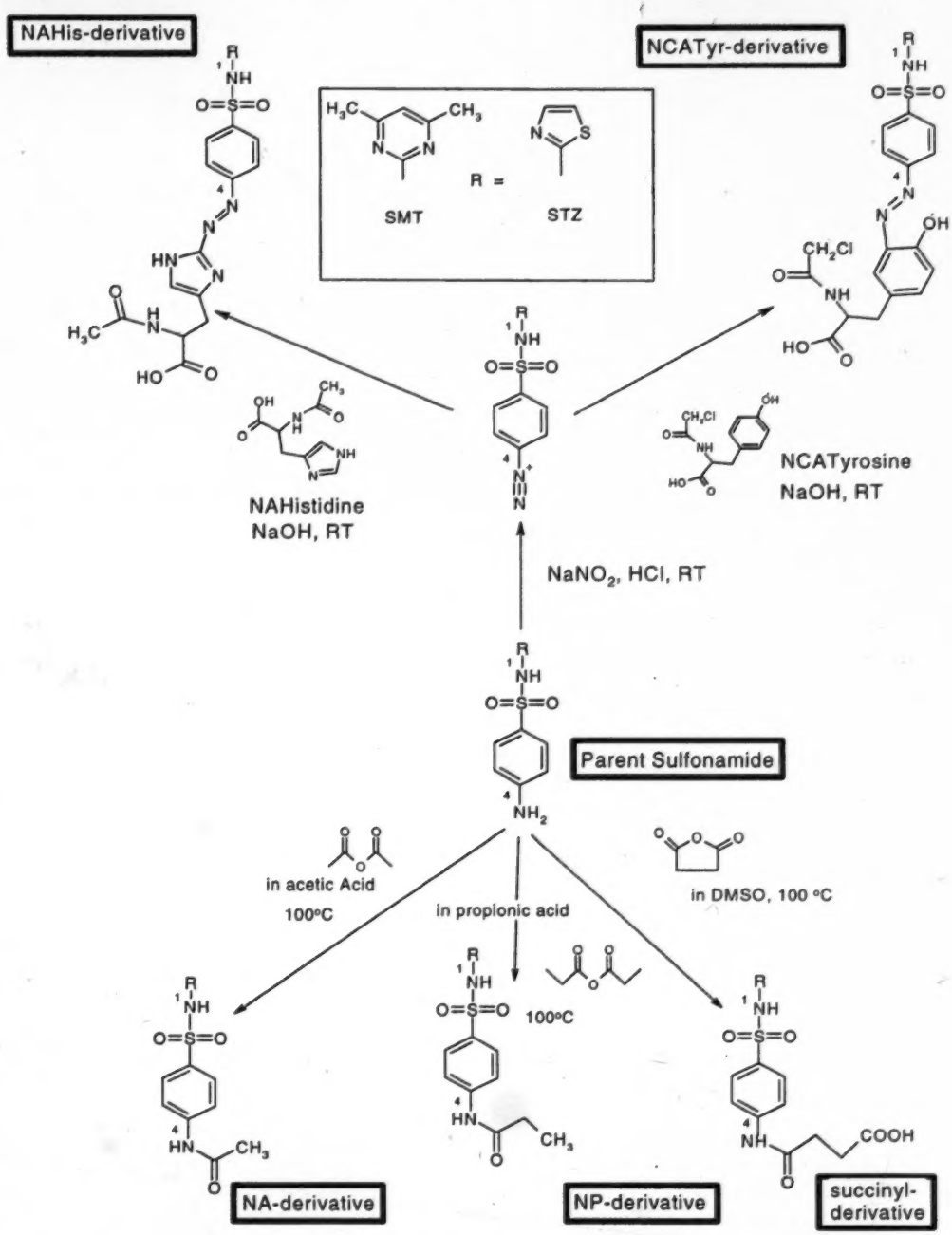

Figure 3.2. Synthesis of N^4-acetyl (NA-sulfonamide), N^4-propionyl (NP-sulfonamide), N^4-succinyl (succinyl-sulfonamide), N-Chloroacetyl-L-tyrosine (NCATyr-sulfonamide) and N-α-Acetyl-L-histidine (NAHis-sulfonamide) derivatives of sulfamethazine (SMT) and sulfathiazole (STZ). RT=room temperature.

an extensive amount of data, and are documented for each antibody competition in the appendices (Chapter 9). Although the results are summarized in this chapter, the appendix data gives an overall impression for the subtleties in both the variety and trends that are not obvious from single IC_{50} values alone.

For all 10 rabbit sera, the average IC_{50} for the reference compounds, parent sulfonamides SMT or STZ, are compared in Figure 3.3. The parent sulfonamide IC_{50} values ranged from a low of 8.13 $\pm$ 0.72 nM (serum 2A using SMT) to a high of 892 $\pm$ 53 nM (serum 3A using STZ). The mean IC_{50} values were significantly lower ($p<0.05$) for competitions using sera from N^4-azo vs. N^4-succinyl immunogen system (86 $\pm$ 24 nM vs. 537 $\pm$ 204 nM, respectively).

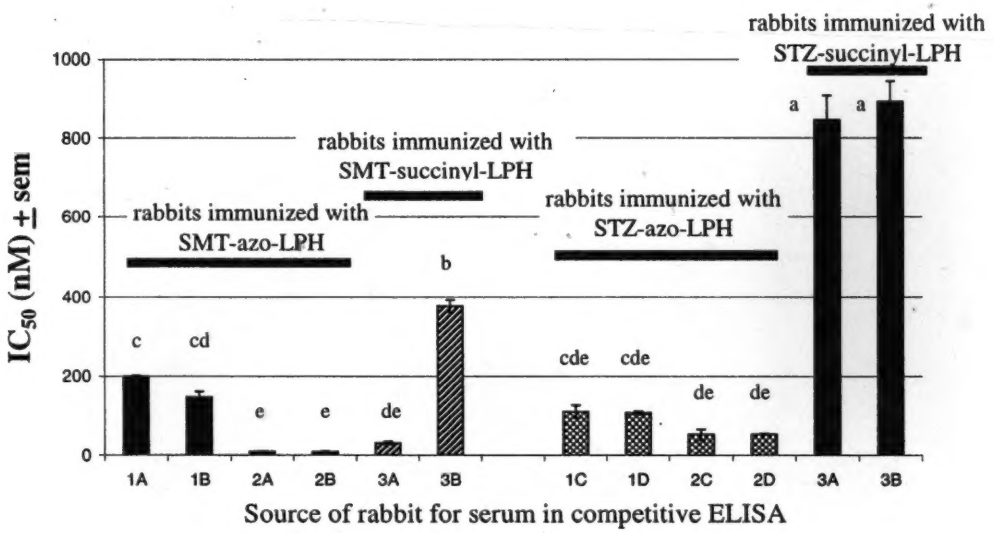

Figure 3.3. IC_{50} ELISA values for the parent sulfonamide for 10 different sera from rabbits immunized with sulfonamide-LPH conjugates.

Mean IC_{50} values ($\pm$ standard error of the mean (sem); n=2) with the same letter are not significantly different ($p>0.05$). IC_{50} is defined as the nM of competitor hapten required to inhibit antibody binding by 50%. Antisera were immunized with sulfamethazine (SMT) or sulfathiazole (STZ) conjugated with azo or succinyl linker arms to *Limulus polyphemus* hemolymph (LPH).

The IC_{50} is a measurement that describes the sensitivity of the assay. It is influenced by both antibody affinity and concentration. The IC_{50} measurement is lower for antibodies with higher affinities, because they are used at lower concentrations to achieve the same standard ELISA response (1.5 at A_{450}). At lower antibody concentrations, each single molecule of competitor ligand has more influence in competing with each antibody molecule. Since these higher affinity antibodies are diluted more in a competitive ELISA, they tend to detect lower concentrations of the competitor sulfonamide being tested (i.e. have lower detection limits). The N^4- azo immunogen sera IC_{50} values were significantly lower than the corresponding N^4-succinyl immunogen, because their titres were relatively higher than the corresponding N^4-succinyl immunogen sera (refer to appendices (Chapter 9) for data on titres and specific antibody concentrations).

The degree of cross-reactivity for each of the five sulfonamide derivatives, relative to the reference compound, was calculated for each of 10 rabbit sera, and is presented in Figure 3.4 as a direct measure of the linker arm effect. The linker arm effect was anticipated to be seen in this experiment, because the sulfonamide derivative competitors all had N^4-amino substitutions which made them more structurally similar to the N^4-linked sulfonamide immunogens used to make the antibodies than the corresponding parent sulfonamide. The linker arm effect was manifested by cross-reactivities significantly greater ($p<0.05$) than 100% relative to the reference parent sulfonamide, SMT or STZ.

As presented in Table 3.2, the average cross-reactivities are summarized by the immunogen type used to make the serum (succinyl- or azo-linker arm), by the sulfonamide type (STZ vs. SMT), and by the competitor derivative type (N^4-acyl substituent vs. N^4-azo-substituent sulfonamide derivatives). The linker arm effect was significant at the 95% confidence level for all sera against all sulfonamide derivatives based on the overall cross-reactivity being significantly greater than 100% compared to the reference parent sulfonamide (744 ± 69% cross-reactivity, n=119). This means that, on average, antibodies bound sulfonamide derivatives with approximately seven fold greater relative affinity than the parent reference sulfonamide.

This linker arm effect was significantly ($p<0.05$) more pronounced for antibodies made from rabbits immunized with STZ-conjugates compared to SMT-conjugates. It is

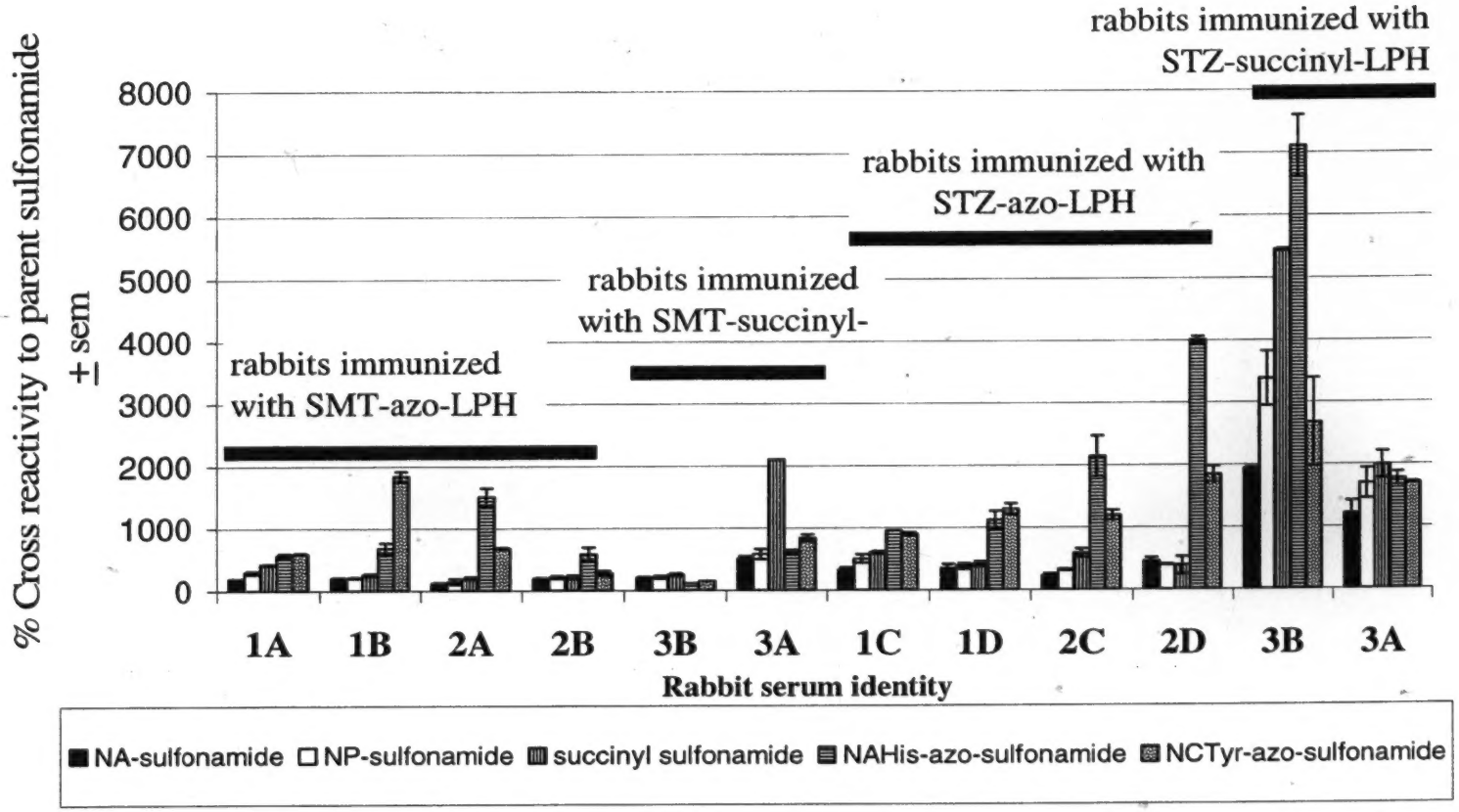

Figure 3.4. Cross-reactivity of 10 different rabbit sera for five sulfonamide derivatives relative to the respective parent sulfonamide in competitive ELISA. The mean cross-reactivity is reported determined b ELISA (Format 3) (± standard error of the mean; n=2). Cross-reactivity is defined as IC_{50} parent sulfonamide /IC_{50} competitor, where IC^{50} = nM of competitor hapten required to inhibit antibody binding by 50%. Competitor cross-reactivities are indicated as different patterned bars, and represent various N^4 – sulfonamide derivatives that mimic the immunogen's sulfonamide and associated N^4-azo or N^4-succinyl linker arm.

Table 3.2. Summary of average competitor % cross-reactivity as described by immunogen sulfonamide type, immunogen linker-type, and sulfonamide competitor linker-type in ELISA

Antibody		Sulfonamide competitor	% Cross-reactivity[1]			
Sulfonamide in Immunogen	Immunogen Linker arm Type[2]	Linker arm Type[3]	Mean		Standard error of the mean	n
SMT	Azo	Azo and Acyl	473	±	71	40
STZ	Azo	Azo and Acyl	923	±	142	40
SMT and STZ	Azo	Acyl	320	±	19	48
SMT and STZ	Azo	Azo	1264	±	160	32
SMT and STZ	Azo	Azo and Acyl	698	±	82	80
SMT	Acyl	Azo and Acyl	561	±	136	19
STZ	Acyl	Azo and Acyl	2889	±	429	20
SMT and STZ	Acyl	Acyl	1629	±	324	23
SMT and STZ	Acyl	Azo	1870	±	572	16
SMT and STZ	Acyl	Azo and Acyl	1725	±	293	39
SMT	Azo and Acyl	Azo and Acyl	502	±	64	59
STZ	Azo and Acyl	Azo and Acyl	1578	±	207	60
SMT and STZ	Azo and Acyl	Acyl	757	±	128	71
SMT and STZ	Azo and Acyl	Azo	1466	±	215	48
SMT and STZ	Azo and Acyl	Azo and Acyl	744	±	69	119

[1] Percent cross-reactivity is defined as the relative percent ratio of the parent sulfonamide IC_{50} relative to the competitor IC_{50}. ELISA Format 3 was used to determine the cross-reactivity relative to the parent sulfonamide. Of the 120 complete competition curves, percent cross-reactivity results were grouped by the logical ideas of sulfonamide type in the immunogen, immunogen linker-type, or sulfonamide competitor linker-type.

[2] Immunogen linker type refers to the different the linkage type, N^4-acyl (succinyl) or N^4-azo, used to link the sulfonamide to LPH to make the immunogen.

[3]Competitor ligands are the derivatives of the parent sulfonamides, sulfamethazine (SMT) or sulfathiazole (STZ), classified as either the acyl group (NA-sulfonamide, NP-sulfonamide, or succinyl-sulfonamide), or the azo group (NAHis-sulfonamide or NCATyr-sulfonamide).

possible that a greater linker arm effect is associated with a smaller hapten like STZ (MW=256) compared to SMT (MW=278), because the linker arm represents a greater proportion of the epitope for the smaller hapten. Consequently, a smaller hapten's derivatives have proportionally more contact within the antibody binding groove compared to the hapten alone, and therefore is stabilized better than a larger analogous hapten system. It is possible the hapten-size effect may be responsible for affinity differences observed between parent sulfonamide and the corresponding derivatives for STZ and SMT. It appears that the degree of the linker arm effect may be a function of the core hapten size (i.e. STZ < SMT), but more research is required to understand if this due to some other differences between STZ and SMT.

When considering all competitors, the linker arm effect was significantly more pronounced ($p<0.05$) for antibodies made from the N^4-succinyl versus the N^4-azo immunogen systems when examining both derivative types (N^4-acyl and N^4-azo). However, there was no significant difference ($p>0.05$) between antibodies of the two linker arm immunogen systems when cross-reactivities were compared relative to competitors possessing the same linker type as the corresponding immunogen (i.e. N^4-succinyl derivatives with N^4-succinyl immunized antibodies versus N^4-azo derivatives with N^4-azo immunized antibodies). This means that by this measurement system, the azo and the succinyl immunogen systems produce antibodies with the same degree of specific linker arm effect.

Also, when considering all antibodies together, the overall linker arm affect was more pronounced in the azo than the acyl competitor type ($p<0.05$). However, when examining a given antibody group by immunogen type, a competitor bias was only statistically significant for antibodies made from N^4-azo immunogen system (4 fold greater cross-reactivity of azo vs. acyl competitors). Structurally more rigid linker arms used in hapten-protein immunogens may produce antibodies that are more specific for that particular hapten-linker arm complex. The double bond of the azo linkage makes it more structurally rigid than the succinyl linkage. It is hypothesized that the greater specificity of the N^4-azo immunogen system may be due to the relatively more rigid nature of the azo-linkage compared to the succinyl linkage. The anti-sulfonamide-succinyl-LPH antibodies were raised against an antigen containing a flexible linker arm,

possibly resulting in the ability to equally recognize and accommodate several sulfonamide N^4 derivative structural shapes. More research is required to understand if the degree of antibody linker-specificity is determined this way.

In this thesis research, the findings confirm the implications of the N^4-azo end of the sulfonamide immunogen being involved in the epitope[1]. The degree of linker arm effect was thought to be able to differentiate the derivatives of a given type by their relative affinity, and in so doing, was expected to better define the size of the sulfonamide epitope on the immunogen. It was first anticipated that antibodies would have higher affinities to the derivatives within their own linker arm group that best approximated their corresponding immunogens. The derivatives within a group all had in common the N^4-azo linkage or the first 2 carbons of the amide bond in the N^4-succinyl linkage. The N^4-acyl derivatives (NA-sulfonamide, NP-sulfonamide, and succinyl-sulfonamide) were considered to be differentiated by their relative lengths after the linker arm (Figure 2.3). Highest affinity for antisera from sulfonamide-succinyl-LPH immunization system was expected to succinylsulfonamide, because it best approximated the structural length of the succinyl linkage. The N^4-azo derivatives (NAHis-sulfonamide and NCATyr-sulfonamide) were considered to be differentiated by amino acid substituent structural character. Garden et al. (1994) demonstrated that SMR-BSA azo linked conjugates were attached to histidine residues almost 3 fold more than to tyrosine residues, so a greater affinity was expected toward the NAHis-sulfonamide derivatives. However, antibodies raised from immunogens of either linkage systems did not significantly ($p>0.05$) differentiate among derivatives within a given type (i.e. within the N^4-acyl group or within the N^4-azo group of derivatives). The results are consistent with

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1 Recently Muldoon et al. (1999) also documented a linker arm effect, when they performed competitive ELISA experiments using monoclonal antibodies raised from mice immunized with sulfadimethoxine (SDM) conjugated to Keyhole limpet hemocyanin by a N^4-azo-linkage. In testing various competitor sulfonamides and derivatives, they concluded that the N^4-azo linkage may be a dominant binding epitope for some antibodies. The N^4-acetyl derivative of SDM had a cross-reactivity > 5 fold that of the parent drug, SDM. Also, they were able to show that sulfapyradine (SPD) had no measurable cross-reactivity in their system, whereas a N^4-azo derivative of SPD had a cross-reactivity of 4.1%. Muldoon's group also did molecular modeling to determine minimum energy conformations of SDM and the related sulfonamides, including SMT and STZ. The lowest energy conformations were bent and were much shorter molecules than the higher energy coplanar structures (4.63 Å vs. 12.97 Å, respectively, for SDM). They suggested the bent structures would have generated antibodies from N^4-azo immunogens that were more sensitive to changes at both the N^4- and the N^1- ends of the molecule, including the linker arm.

an interpretation that antibody specificity to N^4 sulfonamide derivatives is defined and restricted to the first few carbons of the analogous sulfonamide immunogen linkage group adjacent to the sulfonamide. It is proposed that the functional sulfonamide-immunogen epitopes may be no larger than the sulfonamide and its N^4-azo linker arm for the azo-immunization system, or no larger than the sulfonamide and the first 2 carbons of the amide bond for the succinyl immunization system.

Based on this estimate for hapten size, a prediction could be made for the average binding site dimensions of anti-SMT or -STZ polyclonal antibodies. It was assumed that the N^4-linked sulfonamide protein immunogen conjugate was presented to the immune system on end and in a bent sulfonamide conformation as demonstrated by earlier modeling studies (Muldoon et al., 1999) (Figure 3.5).

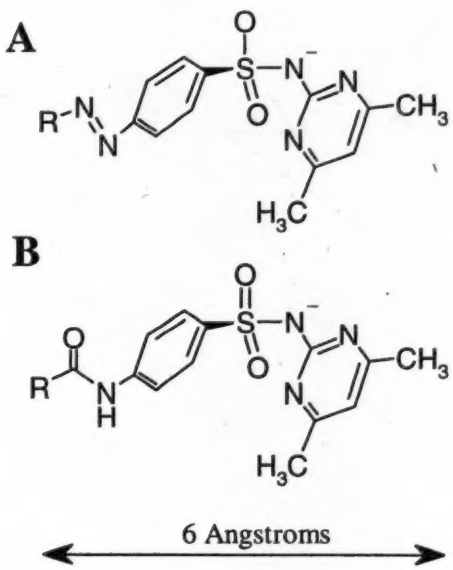

Figure 3.5. Hypothesized hapten dimensions and sizes for azo-linked (A) and succinyl-linked (B) haptens. Based on competitive ELISA results from section 3.3.1 and the lowest energy conformation of sulfonamides (Muldoon et al., 1999).

The average sulfonamide binding pocket is predicted to be narrow and relatively deep, with dimensions of approximately 6-7 Å deep and 3-4 Å across (the same dimensions as the bent sulfonamide structure, including part of the linker system). Also, it is predicted that the binding site domains of the highest affinity anti-sulfonamide antibodies possess at least one positively charged amino acid, either lysine(s) and/or arginine(s), in one or more of the six hypervariable amino acid sequences. Under the physiological conditions that existed during the immunological selection of antibody-producing cells specific for sulfamethazine, the sulfonamide would have had a significant negative character (pK_a of the N_1-nitrogen of sulfamethazine = 7.4, so it would be 50% deprotonated at pH 7.4)[1].

A parallel competitive ELISA experiment, comparing serum and sulfonamide-specific immunopurified antibodies, was performed to demonstrate if immunopurification affected relative antibody specificity among sulfonamide derivatives. For each antibody batch examined, there was no significant average effect of immunopurication except for the parent sulfonamide competitors in batch 1 antibodies ($-28.3 \pm 8.0\%$, $n=4$; $p< 0.05$). The details of this experiment are described in the appendices (Chapter 9).

3.3.2 ELISA Experiment 2: Evaluation of a Linker arm Effect Using Indirect Competitive ELISA by Varying the Type of Coating Antigen

It was desirable to establish a competitive ELISA format that could better approximate the true relative linker arm effect seen in antibodies made using sulfonamide-LPH immunogens linked in different ways. Immunogens used to raise antibodies and coating antigens used in ELISA were made of sulfonamide-protein conjugates linked in the same ways (azo- and succinyl-linkages). Examining different sulfonamide-protein conjugate types (sulfonamide-azo-BSA vs. sulfonamide-succinyl-BSA) in ELISA was thought to more accurately approximate the relative linker arm effect, than by studying different sulfonamide derivatives (not linked to proteins), as was performed and reported in section 3.3.1. It made sense to compare an antibodies' ability

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[1] The sulfonamide would theoretically be stabilized by ionic attraction with the strategic placement of a positively charged amino acid substituent in the hypervariable antibody region. This would be consistent with the observed acidic conditions necessary for easy sulfonamide elution from IAC. Future work to prove these predictions would require the generation of anti-sulfonamide monoclonal antibodies, which could be sequenced and studied with X-ray crystallography, with and without the presence of sulfonamide haptens and derivatives.

to bind two different sulfonamide-BSA coating antigens, distinguished by how their sulfonamides were linked to the BSA (azo- versus succinyl-linked). The exact concentration and binding-availability of the sulfonamide epitopes of sulfonamide-BSA conjugates could not be controlled and made equivalent, so any experiment comparing the relative binding of an antibody to plates coated with sulfonamide-azo-BSA versus sulfonamide-succinyl-BSA, was relatively meaningless. Alternatively, a more meaningful experiment was conceived where different antibodies were allowed to bind the same set of plates pre-coated with succinyl- or azo-linked conjugates, and then were challenged by a competition with the corresponding parent sulfonamide. An antibody with a greater relative linker arm effect to the coating antigen would require a relatively higher concentration of the reference competitor sulfonamide to be displaced from binding the plate. Thus, the relative IC_{50} values of the two antibodies to a given competitor sulfonamide would be a measure of the relative linker arm effect, and was called the relative Acyl/Azo Immunogen IC_{50} quotient[1] (Equation 2.11 described in Chapter 2).

ELISA Format 4 was designed so that the coating antigen concentrations were the same for different analogous immunopurified antibodies (derived from either sulfonamide-succinyl-LPH or sulfonamide-azo-LPH), and the antibody dilutions were adjusted to achieve a limiting antibody environment under standard competitive ELISA conditions, so the antibody would be responsive and sensitive to the competitor hapten. Limiting antibody dilutions were determined by standard two dimensional checkerboard dilutions of both coating antigen and of antibody, as described in Chapter 2, section 2.7.1. The IC_{50} values were determined for the corresponding parent sulfonamide for each competition, using immunopurified antibodies 3B (anti-SMT-succinyl-LPH) and 2A (anti-SMT-azo-LPH) for the anti-SMT system , and 3A (anti-STZ-succinyl-LPH) and 2B (anti-STZ-azo-LPH) for the anti-STZ system.

These results compare the relative linker arm effect for anti-sulfonamide antibodies made with either azo-linked or succinyl-linked immunogens. Unlike free

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[1] *Acyl/Azo Immunogen IC_{50} Quotient* = A/B, where A = IC_{50} antibody associated with the sulfonamide-succinyl-LPH immunogen; B = IC_{50} of antibody associated with the sulfonamide-azo-LPH immunogen.

sulfonamide-derivative ELISA experiments, the ELISA Format 4 demonstrated that there was a significant ($p<0.05$) relative linker arm effect for antibodies derived from succinyl linker arm immunizations (sulfonamide-succinyl-LPH), but not for antibodies derived from azo linker arm immunizations (Table 3.3). This effect was seen for both STZ and SMT sulfonamide test systems as measured by the Acyl/Azo Immunogen IC_{50} quotient. This quotient factor means that if a linking arm effect exists to the sulfonamide-protein conjugates, it is relatively more pronounced for the succinyl linking arm system by a factor of five.

However, the earlier competitive ELISA work in this chapter (section 3.3.1), which used different competing sulfonamide derivatives to measure the linker arm effect, showed that antibodies from the succinyl system did not differentiate sulfonamide derivatives, whereas antibodies from the azo system had a large preference for azo type derivatives. The discrepancy between the two experiments may be rationalized by examining the degree to which each has tested the true structure of the immunogen linker arm. Soluble sulfonamide competitor derivatives do not approximate the structure or environment of the sulfonamide's linker arm attachment to the immunogen (as in section 3.3.1), to the same degree as do the sulfonamide-BSA coating antigen conjugates of ELISA Experiment 2 (section 3.3.2). Free hapten derivatives with linker arm attachments are very different than haptens bonded with the same linker arms to BSA and then immobilized to the surface of an ELISA plate. Also, antibodies likely have very different binding dynamics to soluble sulfonamide competitor ligands than compared to the corresponding sulfonamide hapten linked to a protein immobilized by hydrophobic interaction on an ELISA plate. The measurement system of ELISA Experiment 2 is likely a more valid comparison of the antibody linker arm effect between the two different immunogen systems. Regardless, ELISA Experiment 1 was relevant in evaluating the actual linker arm effect as it would be practically seen when using the antibody to detect or clean-up sulfonamides and their N^4-acetylated metabolites.

Table 3.3. Use of competitive ELISA to measure the relative linker arm effect for antibodies raised with different immunogen linker arms

Sulfonamide Type[1]	Linker Arm Type[2] of Sulfonamide-LPH Conjugate Immunogen	Linker Arm Type[2] of BSA Coating Conjugate	IC_{50} using same sulfonamide type ± standard error of the mean (n=2)	Acyl/Azo IC_{50} Quotient[3]
SMT	Acyl	Azo	15.0 ± 1.3 a	
SMT	Azo	Azo	9.1 ± 0.5 a	1.7
SMT	Acyl	Acyl	18.4 ± 3.2 b	
SMT	Azo	Acyl	3.2 ± 0.1 a	5.8
STZ	Acyl	Azo	122 ± 9 a	
STZ	Azo	Azo	137 ± 21 a	0.9
STZ	Acyl	Acyl	213 ± 1 a	
STZ	Azo	Acyl	47.0 ± 2.0 b	4.5
SMT and STZ		Azo		1.3 ± 0.7 *
SMT and STZ		Acyl		5.2 ± 0.4 *

[1]Sulfonamide type, sulfamethazine (SMT) or sulfathiazole (STZ), was used consistently as the competitor ligand, the sulfonamide in the immunogen, and the sulfonamide in the BSA coating antigen for competitive ELISA Format 4.

[2]The linker arms used for immunogen (LPH-sulfonamide) or coating antigen (BSA-sulfonamide) protein conjugates were either an acyl linkage or an azo linkage.

[3]Acyl /Azo Immunogen IC_{50} Quotient = A/B, where A = IC_{50} antibody associated with the sulfonamide-succinyl-LPH immunogen; B = IC_{50} of antibody associated with the sulfonamide-azo-LPH immunogen. * Acyl /Azo Immunogen IC_{50} quotients were averaged for SMT and STZ

a-b Mean IC_{50} values that have the same letter within a competition pair are not significantly different ($p>0.05$).

3.3.3 ELISA Experiment 3: Cross-reactivity of Related Sulfonamides and Derivatives using an Immunopurifed Antibody in Competitive ELISA

An experiment was designed to establish the cross-reactivity of sulfonamides structurally related to SMT in competitive ELISA using immunopurified antibody 1A. These results were important for later correlation to relative recovery, capacity, and competition experiments in IAC research (Chapter 4). Sulfonamides similar in structure to SMT (sulfamethazine, sulfanilamide, sulfamerazine, sulfadiazine, sulfapyradine, sulfadimethoxine, sulfathiazole, N^4-acetylsulfamethazine, N^4-propylsulfamethazine, N^4-acetylsulfathiazole, N^4-propylsulfathiazole) were serially diluted to a maximum of 10^8 from their respective 3.6 mM standards, and then these dilutions were compared for their ability to inhibit a limited amount of anti-SMT-azo-LPH 1A immunopurifed antibody from binding the coating antigen, SMT-azo-BSA, in indirect competitive ELISA (Format 3). The analogous data from section 3.3.1 for immunopurifed antibodies from serum 1A in competition with N^4-succinylsulfamethazine, N^4(N-α−acetyl-L-histidine)-SMT derivative, and N^4(N-chloroacetyl-L-tyrosine)-SMT derivative were also included with this data set.

This competitive ELISA experiment was able to examine the large differences of the 1A antibody affinities for these different sulfonamides (Figure 3.6). Overall, the shape of each competition function is very similar, implying that antibody-hapten dynamics responsible for the inhibition are similar for each sulfonamide. The IC_{50} for each sulfonamide was determined and the cross-reactivity of each sulfonamide was expressed in relation to SMT (% cross-reactivity = 100 x IC_{50} SMT / IC_{50} sulfonamide competitor) (Figure 3.7). The cross-reactivities of SMT-related sulfonamides followed a pattern. As documented and discussed in section 3.3.1, all SMT derivatives had significantly greater than 100% cross-reactivity, as explained by the linker arm effect. All sulfonamides that were not derivatives of SMT, as expected, had significantly less than 100% cross-reactivity relative to SMT. The sulfonamides that were more similar in structure to SMT generally had higher cross-reactivities than other sulfonamides that were less similar. Sulfamerazine (SMR), the structurally closest sulfonamide to SMT and differentiated by having only one less methyl on the pyrimidyl substituent, had the highest cross-reactivity of the non-SMT type sulfonamides (18.8 ± 1.1%). Next,

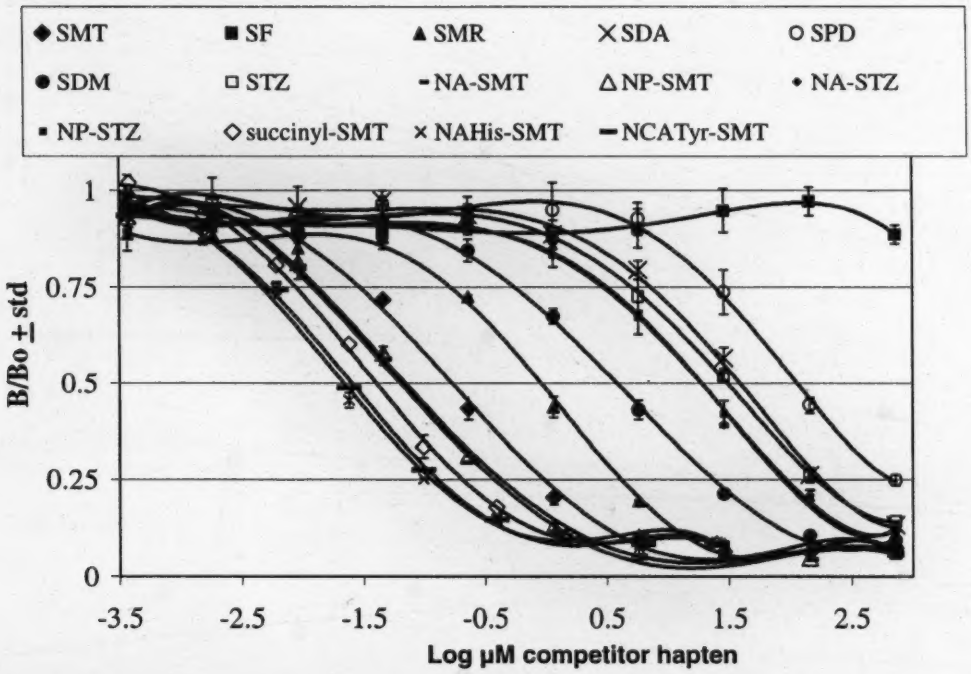

Figure 3.6. Inhibition of anti-SMT immunopurified antibody 1A by of sulfonamide competitors in ELISA

Competitive indirect ELISA Format 3 was used with plates coated with sulfamethazine-azo-bovine serum albumin. Mean values reported ± standard deviation. Where no error bars are visible, they are smaller than the symbol.

Competitor analogues and derivatives used were sulfamethazine (SMT), sulfanilamide (SF), sulfamerazine (SMR), sulfadiazine (SDA), sulfapyridine (SPD), sulfadimethoxine (SDM), sulfathiazole (STZ), N^4-acetylsulfamethazine (NA-SMT), N^4-propionylsulfamethazine (NP-SMT), N^4-acetylsulfathiazole (NA-STZ), N^4-propionylsulfathiazole (NP-STZ), succinylsulfamethazine (succinyl-SMT)*, N^4(N-chloroacetyl-L-tyrosine-sulfmethazine (NCATyr-SMT)*, N^4(N-α−acetyl-L-histidine-sulfamethazine (NAHis-SMT)*.

*The competitor curves for these IC_{50} were obtained from section 3.3.1.

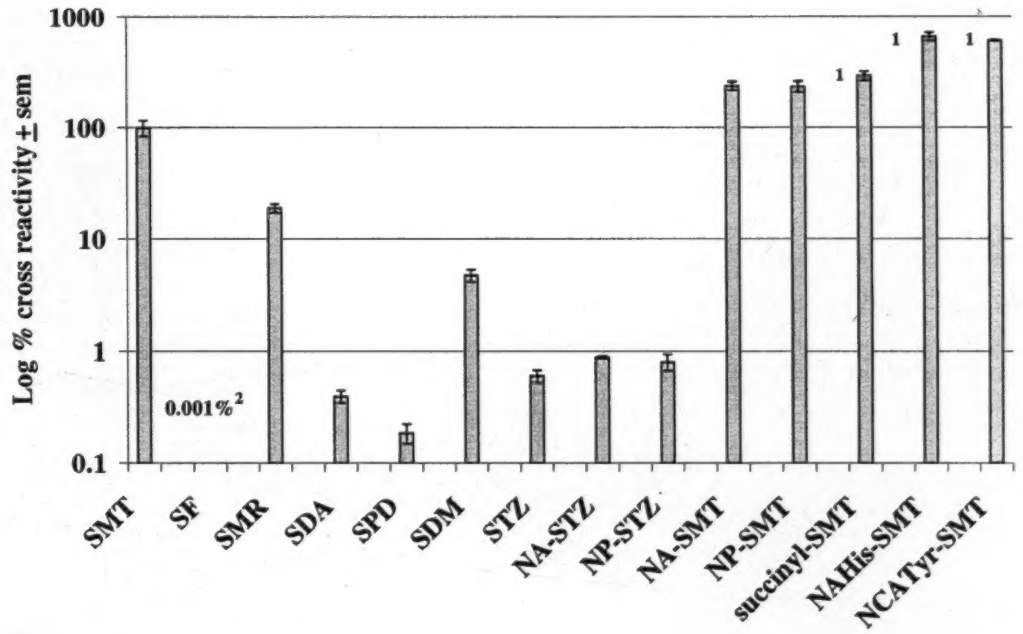

Figure 3.7. Cross-reactivity of various sulfonamides in competitive ELISA relative to SMT using 1A anti-SMT antibodies

Percent cross-reactivity = 100 x IC_{50} SMT / IC_{50} sulfonamide competitor. Competitive indirect ELISA Format 3 using SMT-azo-BSA coating antigen. Means reported ± standard error of the mean (sem) (n=2).

[1]The competitor curves for these IC_{50} were obtained from section 3.3.1.

[2]Since the sulfanilamide competition only effected a 10% inhibition of antibody binding at the highest competitor concentration, the sulfanilamide cross-reactivity relative to SMT based on a comparison of their IC_{10} values, and was estimated to be 0.001%.

Competitors used are sulfamethazine (SMT), sulfanilamide (SF), sulfamerazine (SMR), sulfadiazine (SDA), sulfapyridine (SPD), sulfadimethoxine (SDM), sulfathiazole (STZ), N^4-acetylsulfamethazine (NA-SMT), N^4-propionylsulfamethazine (NP-SMT), N^4-acetylsulfathiazole (NA-STZ), N^4-propionylsulfathiazole (NP-STZ), succinylsulfamethazine (succinyl-SMT)*, N^4(N-chloroacetyl-L-tyrosine-sulfmethazine (NCATyr-SMT)*, N^4(N-α−acetyl-L-histidine-sulfamethazine (NAHis-SMT).

sulfadimethoxine (SDM), a larger sulfonamide compared to SMT, has similar overall shape, but likely owing to the long ether extensions of the methyl groups, had still lower cross-reactivity (4.76 ± 0.38%). The absence of both methyl groups in sulfadiazine (SDA) compared to SMT, was likely responsible for its lower cross-reactivity (0.398 ± 0.034%). The pyramidal ring of SDM is attached differently than SMT, SMR, or SDA relative to the two ring nitrogens (see Figure 1.2 of Chapter 1). These results imply that the correct positioning of both pyramidal ring nitrogens is apparently not as critical for antibody binding as are methoxy groups present in SDM. However, it appears that the arrangement or presence of nitrogen atoms in the pyrimidal ring play an important role, because sequential elimination of these in the competitor hapten structure significantly reduces the cross-reactivity, as is seen for SDA (0.398 ± 0.034%), and then sulfapyradine (SPD) (0.187 ± 0.027%). As demonstrated by sulfathiazole's (STZ) greater cross-reactivity than SDA (2.22 ± 0.05% vs. 0.398 ± 0.034%, respectively), the six-membered benzene shape is not as critical as is the electronic configuration of the heterocyclic functional group substituted on the N^1 position of the sulfonamide. STZ has a sulfur and nitrogen on opposing sides of its heterocyclic 5-membered ring structure that may be electronically similar to the two nitrogens in the heterocyclic 6-membered ring structure of SMT, and ultimately, important in stabilizing the antibody-hapten interaction[1].

Sulfanilamide (SF) caused almost no inhibition of antibody binding. The extremely low affinity of the anti-SMT antibody to sulfanilamide may be mainly due to sulfanilamide's smaller size compared to SMT, and there are also some important electronic differences between the two compounds to be considered in causing this effect.

3.4. SUMMARY

Sulfonamides, STZ or SMT, were covalently linked to LPH with azo-linker or succinyl-linker arms, to make 4 different immunogens used to immunize a total of 10

[1] It is interesting to note that the N^4-acetyl and the N^4-propionyl derivatives of STZ had higher ($p<0.05$) cross-reactivities relative to SMT than did STZ (0.885 ± 0.017%, 0.801 ± 0.134%, and 0.604 ± 0.052%, respectively). Even though antibody 1A was made against an SMT-azo-LPH immunogen and not STZ, the N^4-acyl groups of STZ bound the antibody better than their parent sulfonamide, STZ. This again exemplifies the impact of the linker-arm of the immunogen to confer greater specificity to compounds that possess linker-type attachments in the appropriate position.

rabbits. The sera from the N^4-azo immunogen system had the highest titres and produced the most sulfonamide-responsive ELISAs as measured by the parent sulfonamide IC_{50} values. Immunopurification of antibodies using sulfonamide-BSA enrichment columns was shown to be able to enrich the sulfonamide-specific fraction by 4 to 6 fold, virtually eliminate ELISA cross-reaction to LPH and BSA controls, and have no significant overall effect on competitive ELISA performance (data from the appendix, Chapter 9).

Competitive ELISA experiments demonstrated that a significant linking arm effect did exist for all antibodies. The antibodies on average had at least a 7 fold preference for sulfonamide derivatives that had linker-type attachments. This phenomenon was more significant for the antibodies raised by N^4-succinyl immunogen system, which did not differentiate the two linker arm types. On the other hand, antibodies raised by the N^4-azo immunogen system had a 4 fold preference for their own linker N^4-azo type over the N^4-acyl type. Based on the goal of this thesis, antibody linker arm effects were seen as negative because the subsequent antibodies used in IAC may not have equivalent affinity to SMT and its related N^4-acyl type metabolite, NA-SMT. However, it was indicated that the relatively lower linker arm affinity of antibodies raised by the N^4-azo immunogen system for N^4-acyl type derivatives (like NA-SMT), made them the best candidates for the equivalent IAC recovery for both SMT and NA-SMT.

Antibodies raised from immunogens of either linkage systems did not significantly differentiate between sulfonamide derivatives within a given type (i.e. the N^4-acyl or N^4-azo derivatives). From this finding it was proposed that antibody specificity to N^4 SMT derivatives may be defined and restricted to an epitope described by the sulfonamide itself together with the first few carbons of the analogous sulfonamide immunogen linkage group adjacent to the sulfonamide. This may be important in considering the design of the sulfonamide-protein conjugate for use as an immunogen, ELISA coating conjugate, or in antibody enrichment columns.

Competitive ELISA was used to resolve a 10^5 fold affinity spread among fourteen related sulfonamides and derivatives. The range of cross-reactivities described by this experiment helped to establish what average structural features of the SMT molecule were important in its stabilization by an anti-SMT antibody. For example, for the antibody 1A made against SMT, the structural features within the pyramidal ring, like the

positioning of the nitrogens, were less important that the presence of methyl moieties. This experiment and the other parts of this chapter represented the specific foundation of knowledge and antibody materials to make and evaluate IACs made from anti-SMT antibodies in subsequent chapters. Insights were gained about factors that affect the cross-reactivity profile of a polyclonal serum with respect to the competing hapten derivatives, the immunogen design, the design of the immunopurification enrichment column, and the subsequent elution conditions of that column.

The general goal of this chapter was met; antibodies were developed and characterized for later correlative work with corresponding IAC systems. The practical objectives were also met; batch 1 anti-SMT sera, 1A and 1B, raised from the N^4-azo immunogen system, were selected as the best candidates for purification and future use in IAC clean-up of SMT from environmental samples. These antisera represented the overall best compromise in possessing high titres and specific antibody yields, good specific affinity for SMT, and a low linker arm bias for the N^4-acyl attachment of the NA-SMT metabolite.

Chapter 4

DEVELOPMENT AND CHARACTERIZATION OF SULFONAMIDE-SPECIFIC IMMUNOAFFINITY COLUMNS

4.1. INTRODUCTION

4.1.1. Development of a Immunopurification System for Sulfonamide Clean-up

Ideally, a one-step procedure to purify and concentrate trace-level sulfonamides from environmental samples was needed to complement the potential analytical efficiency of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Compared to other mass spectroscopy (MS) systems that need both liquid-liquid extraction, then solid phase extraction, and then high performance liquid chromatography (HPLC) or gas chromatography (GC) isolation before analysis, MALDI-TOF MS is inherently more tolerant of sample impurities, and has the capability of high through-put. However, because environmental samples are so complex, significant purification is necessary, especially to detect concentrations of drugs at parts per billion levels. Conventional reverse solid phase extraction systems such as C_{18}, which work on the principle of hydrophobic interaction, were found not to be specific enough to adequately purify sulfonamides from environmental samples. A simple and efficient clean-up step was considered feasible if a more specific means of extraction were used.

An anti-sulfonamide immunoaffinity column (IAC) system was proposed to be sufficient to prepare sulfonamide residues adequately prior to MALDI-TOF MS. This chapter presents the results of three IAC experiments which characterizes their operational ranges and limitations. The immunopurifed antibodies used in these IACs were already described by ELISA in Chapter 3.

4.1.2. The Ideal Purification System

Several considerations guided the design of experiments to evaluate and characterize IAC systems as they were developed, and were inspired by thinking about the ideal purification system. An ideal purification system...

1. ...is specific for the compounds of interest.
2. ...is able to extract an internal standard equivalently.
3. ...has 100% recoveries for the compounds of interest.
4. ...has adequate column capacity with respect to capacity to bind the analyte.

5. ...has identical affinity for each compound of interest and therefore shows no recovery biases under column saturation dynamics.

6. ...is able to extract compounds of interest in one, simple application step and then also elutes them in one, simple, low volume step.

7. ...has a column size and elution conditions such that the column's elution volume is small enough to concentrate the compounds of interest adequately for the lowest concentrations required for detection.

8. ...is resistant to sample "breakout" relative to the sample volumes applied[1].

9. ...is durable and reusable, and is able to be regenerated fully for subsequent extractions.

4.1.3. Theoretical Considerations of Sample Volume, Sample Concentration and Purification

Several practical considerations, regarding sample volume and concentration, guided the initial design of the IAC system. MALDI-TOF MS is capable of detecting many sulfonamide compounds to a lower limit of about 200 ng/L in methanol without concentration (Ling et al., 1998). An initial goal was to detect sulfamethazine and its N^4-acetyl metabolite at concentrations of 1 ng/L in complex environmental samples, because this level represented a common and approximate limit of quantification for other validated ELISA and MS-type procedures. To achieve detection of a 1 ng/L solution by MALDI-TOF MS under ideal conditions would therefore require a minimum of 200 fold concentration to attain its on-probe lower limit of detection (200 ng/L). If the elution volume of the purification system were assumed to be about 4 mL, as it is for most small solid phase extraction (SPE) systems designed for larger volume samples, then a minimum of 800 mL of sample would be required to achieve MALDI-TOF MS detection of sulfonamide directly in the IAC eluant. Thus the project went forward with the assumption that a 1 ng/L sulfamethazine (SMT) solution theoretically concentrated by an

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[1] Sample breakout is a phenomenon experienced by all flow through purification systems, where the compound of interest is in equilibrium with the solid phase, and large application volumes partially elute it due to simple mass action.

IAC system and detected by MALDI-TOF MS, if there were minimal losses of sulfonamide and a high degree of purification.

4.2. CHARACTERIZATION OF ANTI-SULFONAMIDE IACS

Using the polyclonal antibodies already purified and characterized (see Chapter 3), initial IAC development experiments laid down the foundation of knowledge to optimize and understand many of the physical parameters of the immunoaffinity chromatography system to be later used in residue analysis (Chapters 5 and 6). These initial experiments are documented in the appendices (Chapter 9) together with the considerations relevant to the theoretical limitations of IAC performance. The appendices describe the antibody loading capacities achieved on the IAC systems for antibody sources that were protein-G purified or immunopurified, the subsequent sulfonamide loading capacities, and issues regarding sulfonamide elution conditions from the IACs. Problems intrinsic with any liquid-solid chromatographic system were investigated, and compromises in the IAC design were made and discussed to optimize performance. Breakout capacity, column collapse due to hydrostatic pressure, non-specific binding of sulfonamide, steric hindrance considerations, antibody loss or "bleed", and other relevant issues are fully discussed in the appendix.

The development and preliminary evaluation of anti-sulfonamide immunoaffinity columns (IACs) is investigated to achieve greater efficiency in preparing sulfonamides prior to MS analysis. The performances of IACs were compared to the corresponding enzyme-linked immunosorbent assay (ELISA) performances of antibodies documented in Chapter 3. Also, ELISA was evaluated as a polyclonal sera screening tool to assist in choosing antibodies for IAC candidates, and ultimately, for predicting IAC performance.

4.2.1. IAC Experiment 1: IAC Recovery of Equimolar Concentrations of Related Sulfonamides at Various Column Saturation Levels

The goal of this experiment was to determine the relative specificity of an anti-SMT IAC, made with immunopurified 1A antibodies, toward different sulfonamides, including the N^4-acetyl metabolite of SMT, over a range of sulfonamide concentrations.

The ideal IAC system should have similar high recoveries for both SMT and its related NA-SMT metabolite, or under saturation conditions, have identical affinity for each compound and therefore show no recovery preferences due to differences in the chemical structure of SMT and NA-SMT. Although it was primarily of interest to determine if SMT and NA-SMT were recovered similarly, it was also important to evaluate the recovery performance of the IAC toward other sulfonamide analogues that had low cross-reactivity in ELISA (refer to section 3.3.3). This would help to determine if the IAC could recover other sulfonamides structurally different than SMT. The IAC was challenged to simultaneously extract equimolar mixtures of SMT, NA-SMT, SMR, SPD, SDA, and STZ at both sub-saturating and saturating levels. The recovered sulfonamides were eluted, concentrated by a micro-SPE column, and quantified by MALDI-TOF MS in triplicate relative to the established response ratios with respect to an NP-SMT standard added to the IAC elution mixture. The response ratios had been established by directly processing an equimolar mixture of all six sulfonamide standards together with the NP-SMT standard through a micro-SPE column, and then analyzing the eluant by MALDI-TOF MS.

The recoveries are reported as a percentage of the amount added to the IAC (Table 4.1) and as a proportion relative to the other sulfonamides (Figure 4.1) for each of the six concentration treatments. It was assumed that the same IAC saturation capacity for SMT alone (68.7 nmol) could be used to estimate the saturation capacity for the sulfonamide mixture. Under non-saturating conditions, this IAC system showed a remarkable and broad ability to recover all 6 sulfonamide compounds. At 60% of column saturation, there was no significant difference ($p>0.05$) in the recovery of the 6 sulfonamides, except sulfanilamide, which did not bind to the IAC. Under non-saturating conditions, this means that an IAC made specific for the SMT sulfonamide has the potential to be a generic clean-up system for other sulfonamides too. There was a consistent IAC recovery bias between the two antigenically similar sulfonamides, NA-SMT and SMT, over the whole range of concentrations examined, meaning this IAC system would be suitable for the detection of SMT and its main metabolite, regardless of IAC saturation dynamics. Yet, as the IAC extraction was progressively saturated, the

Table 4.1. Percent recoveries[1] of six related sulfonamides[1] loaded simultaneously onto an anti-SMT immunoaffinity column at various saturation levels[2]

	IAC saturation levels[3]					
	30%	**60%**	**150%**	**300%**	**900%**	**3000%**
SMT	40.6 ± 6.5	79.2 ± 8.2	102.5 ± 6.2	59.6 ± 4.4	21.9 ± 4.1	5.6 ± 0.9
NA-SMT	50.5 ± 1.9	61.7 ± 12.2	60.0 ± 19.1	39.5 ± 2.0	16.7 ± 1.3	4.9 ± 1.0
SMR	56.1 ± 3.8	103.5 ± 11.5	111.7 ± 12.7	23.2 ± 0.7	6.3 ± 1.3	1.8 ± 1.2
SDA	105.7 ± 15.7	100.7 ± 39.6	3.9 ± 5.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SPD	90.0 ± 48.6	103.8 ± 47.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
STZ	67.4 ± 7.7	100.1 ± 9.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[1] Abbreviations for sulfonamides: sulfamethazine (SMT), N^4-acetylsulfamethazine (NA-SMT), sulfamerazine (SMR), sulfadiazine (SDA), sulfapyradine (SPD), and sulfathiazole (STZ).

[2] Refer to Equations 2.19, 2.20 and 2.21 for determination of recoveries using MALDI-TOF MS. Values are averages ± standard error of the mean or replicate MALDI-TOF MS spectra (n=3).

[3] The saturation levels were determined based on the total sulfonamide IAC loading expressed as a percentage of IAC capacity. IAC capacity was defined as the level of saturation for SMT on this IAC made with 1A immunopurified antibodies (68 nmol). The loading volume was constant at 2 mL, and the equimolar concentration of the six sulfonamides varied to achieve loadings from 30% to 3000 % of IAC saturation.

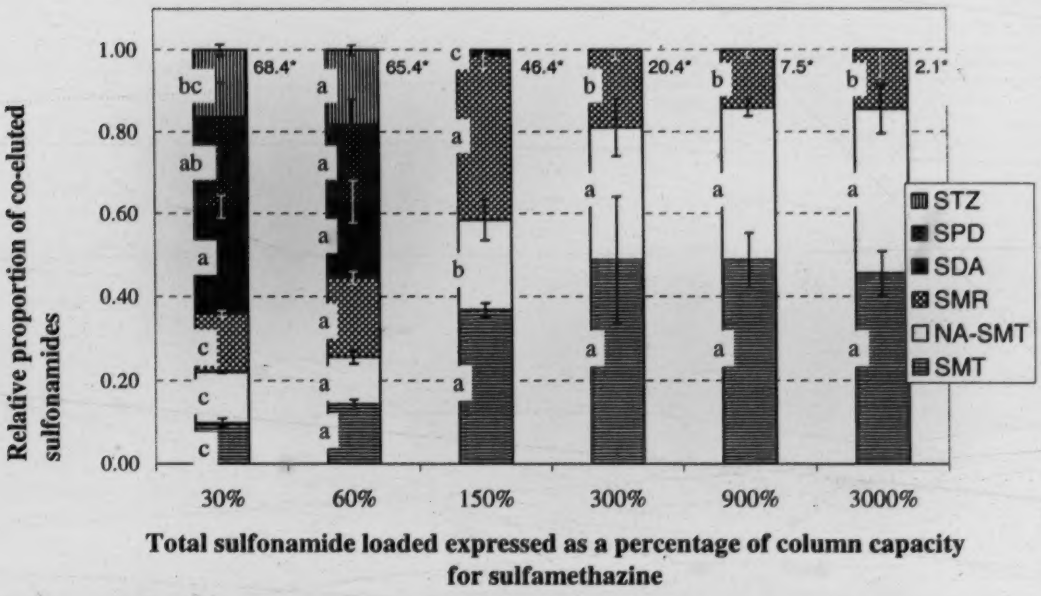

Figure 4.1. Relative simultaneous recovery of equimolar quantities of related sulfonamides from immunoaffinity column 1A. Refer to Equations 2.19, 2.20 and 2.21 in Chapter 2 for determination of relative recoveries sulfamethazine (SMT), sulfathiazole (STZ), sulfapyridine (SPD), sulfamerazine (SMR), and N^4-acetylsulfamethazine (NA-SMT) using MALDI-TOF MS and an NP-SMT internal standard.

a-c Mean relative proportion of co-eluted sulfonamides within a bar that have the same letter are not significantly different ($p>0.05$).

* Numbers (top right of the bars) are the total % recoveries of the combined sulfonamides relative to their loaded amounts.

sulfonamides least similar to SMT and NA-SMT were significantly out-competed to non-detectable levels (SPD & STZ first, and then SDA) by SMT, NA-SMT and SMR. Under highly saturated conditions of 300-3000%, the relative recovery ratios of SMT and NA-SMT were significantly different ($p<0.05$) from SMR under saturation conditions, and were approximately 1:0 : 0.9 : 0.3, respectively.

As shown in Figure 4.1, as long as the total moles of sulfonamide in the mixture did not exceed the IAC's mole saturation point for SMT, very different sulfonamide structural types could be extracted[12]. This can be explained if one assumes high affinity IAC antibodies are not necessary for sulfonamide extraction under low volume sample addition and column washing conditions (total of 25 column volumes). Under non-saturating conditions, where there is little or no competition among sulfonamides for available antibody binding sites, even sulfonamides like STZ (structurally dissimilar from the immunogen sulfonamide) could be recovered at high levels. However, when the sulfonamides were added at concentrations progressively exceeding the saturation point, greater competition existed among the mixed sulfonamides for the now limited antibody binding sites available, and only sulfonamides with the highest average affinities to IAC antibodies could be recovered.

NA-SMT and SMT have the same N1 heterocyclic substituent, and they had near equivalent relative recoveries at 3000% saturation. Yet SMR, the closest structural

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[1] When sulfonamide loadings saturated the IAC by more than 100%, the total combined recoveries of the 6 sulfonamides decreased in proportion to the level of saturation above 100%. It is important to note that the low recoveries of sulfonamides were not due to poor performance of the IAC, but rather due to exceeding of the number of IAC antibody binding sites.

[2] The total amount of mixed sulfonamide extracted by the IAC for each of the four saturated loading levels were similar (45.5 $\pm$ 8.5 nmol), but only represented on average 67% of the value determined for SMT alone (68.7 $\pm$ 1.7 nmol). This discrepancy did not make sense at the time of this experiment, however, possible explanations were provided by the results of section 4.2.3. The original acidic eluant used for the IACs (10 mM HCl, pH 2.5) was developed for IACs 1A and 1D, and was assumed to apply to other IACs made with immunoaffinity antibodies purifed using a pH 2.5 elution system. However, the 10 mM HCl buffer was found to not be adequate to elute some of the derivatives (like NA-SMT) from IAC's made with antibodies 1A or 1C, and the addition of 20% ethanol to the eluant buffer solved the problem. Because only about two thirds of the IAC's lowest affinity antibodies were being used to exchange sulfonamides when the column had been exposed to NA-SMT and was regenerated with 10 mM HCl, the total recoveries were never more than about 0.7 of what was determined previously for SMT alone. Large volumes of 10 mM HCl or the addition of 20% ethanol to the elution buffer did regenerate the column. Regardless, the general conclusions made here about relative antibody recovery of sulfonamides still are valid, and the specific recovery ratios that would have occurred if the column had been fully regenerated are not known.

relation to SMT of all the non-SMT compounds, was the only other sulfonamide that had a measured recovery at saturation levels above 150%. SMR competes for antibody sites because of it structural similarity, and appeared to have reached a constant share of 15% for its occupancy on the IAC under higher saturation conditions. The levelling off to 15% IAC SMR occupancy implies that there is a subpopulation of antibodies that does not differentiate SMR from SMT. If all antibodies had a bias toward the SMT form, then SMR would have eventually been out-competed to zero binding at higher saturation levels[1].

This competitive IAC (section 4.2.1), like the earlier competitive ELISA experiments (section 3.3.3), established a competition between sulfonamides for a limited number of available antibodies. Although the IAC system was less sensitive and could not measure the large range of antibody affinities for different sulfonamides as well as ELISA, the trends were similar. ELISA could be used to predict the ability of a sulfonamide to be recovered in non-saturating conditions, or to withstand competition in saturating conditions (Figure 3.7). Under non-saturating conditions of this particular anti-SMT IAC 1A system, a sulfonamide compound would likely be recovered well if it cross-reacted in the corresponding competitive ELISA within three orders of magnitude of the parent hapten. This is consistent with the observed recoveries of SPD, SDA, STZ, and with the inability of sulfanilamide (SF) to be recovered by the same IAC in IAC Experiment 3 of this Chapter (see section 4.2.3). Furthermore, under saturating conditions, a sulfonamide compound would likely be recovered well if it cross-reacted in the corresponding competitive ELISA within one order of magnitude of the parent hapten (consistent with the recovery of SMR). These findings are important because they help define the operational ranges of an anti-sulfonamide IAC for various types of sulfonamides. Future work may help to understand and establish certain "rules of thumb" applicable for hapten IAC recovery using antibodies raised in defined ways for specific hapten models[2].

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[1] These observations imply that 45% of the heterogeneous polyclonal antibody population (3 sulfonamides X 15% each) effectively does not differentiate SMR, SMT, and NA-SMT, and the remaining 55% of the antibody population likely has relatively greater affinity for SMT.

[2] Recently, a positive but poor correlation (R^2=0.45) has been reported between the log IC_{50} in competitive ELISA to the corresponding IAC percent recovery of haptens (Shelver et al., 2002). Various monoclonal

4.2.2. IAC Experiment 2: IAC Competition of Parent Sulfonamide with Sulfonamide Derivatives under Saturating Conditions

The objective of this research was to investigate if competitive ELISA could predict IAC performance under competitive conditions for sulfonamide derivatives. Specifically, an experiment was designed to compare IAC and ELISA in their abilities to demonstrate competitive differences among the parent sulfonamides (SMT and STZ), and their respective derivatives (N^4-acetyl, N^4-propionyl, N^4-succinyl, and N^4(N-acetyl-L-histidine)). Four IACs (0.4 mL), all from batch 1 immunizations (sera 1A, 1B, 1C, and 1D), were challenged in duplicate with a parent sulfonamide and a single sulfonamide-derivative competitor at equimolar, IAC-saturating concentrations. IACs from antibodies made against SMT or STZ immunogens were challenged to saturating levels[1] of SMT or STZ and their derivatives, respectively. After loading an IAC, they were washed and then eluted with 10 mM HCl. The moles of parent sulfonamide in the eluant were determined using the Bratton Marshall (BM) assay [2]. The competition was measured by the prevention of the parent sulfonamide from achieving full binding capacity on the column. This inhibition could be expressed simply as % inhibition relative to full

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antibodies raised against a polychlorinated dibenzo-p-dioxin (PCDD) hapten model were used to establish their competitive ELISA IC_{50} values, which ranged over three orders of magnitude. Unlike these findings with PCDDs where large differences in IAC recoveries were evident, the IAC recoveries for different sulfonamides here were very similar to each other under similar non-saturating conditions as used in the PCDD research. The IC_{50} values for SMT and SPA were 0.155 ± 0.018 and 82.9 ± 11.7 µM, respectively, and represented a 500 fold difference of affinity, yet under non-saturating conditions both compounds were recovered similarly in IAC.

[1] The total mixed sulfonamide mole loading was at least twice the IAC saturation capacity as determined by the parent sulfonamide alone.

[2] The BM assay could only detect sulfonamides with free aromatic amino groups, thus could not detect the derivatives of sulfonamides; all BM assay responses were therefore attributed to parent sulfonamide.

capacity binding of the parent sulfonamide (Equation 2.6)[1], or also as cross-reactivity (Equation 2.7)[2], which was as a more dynamic measure of inhibition.

Some of the sulfonamide derivatives significantly inhibited their respective parent sulfonamide from binding on the IAC to some extent (Table 4.2). However, the inhibition was not as large as in the analogous competitive ELISA (Experiment 1) (Figure 4.2). NAHis-sulfonamide was the only sulfonamide derivative tested that consistently inhibited the corresponding parent sulfonamide from binding the IAC by more than 50% for all four IACs tested[3].

The greater antibody preference for its respective NAHis-sulfonamide can be explained due to a specific linker arm effect. All batch 1 IAC antibodies were derived from rabbits immunized with sulfonamides linked to LPH amino acid residues via an azo linkage, so a linker arm effect was anticipated in this IAC experiment to the azo type linkage. As with the analogous competitive ELISA experiment, the linker arm effect was demonstrated by relatively higher inhibition of azo-linked sulfonamide derivatives than sulfonamide derivatives with hemisuccinate linker-arms (hemisuccinate, N^4-acetyl, and N^4-propionyl). The positive trend was established correlating cross-reactivity of sulfonamides in competitive ELISA and competitive IAC (Figure 4.3). Competitive ELISA may be used to predict the linker arm effect expected in an analogous IAC system under saturation conditions ($y = 0.1063x + 101.32$; $R^2 = 0.7752$, where y = % cross-reactivity in IAC and x = % cross-reactivity in competitive ELISA).

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[1] Percent IAC inhibition is described by Equation 2.6. = 100*(A-B)/A
A = the maximum capacity of the IAC for the parent sulfonamide, SMT or STZ, when exposed to the IAC in saturating conditions; B = capacity of the column for the parent sulfonamide, SMT or STZ (68.7 $\pm$ 1.78 and 18.9 $\pm$ 3.9 nmol SMT for IACs 1A and 1B, respectively, and 70.5 $\pm$ 1.6 and 38.2 $\pm$ 0.4 nmol STZ for IACs 1C and 1D; note: the IAC volumes were variable and not necessarily equal, but the same IAC for each antibody source was used throughout the experiment).

[2] The cross-reactivity of sulfonamide derivative competing with the parent sulfonamide for IAC binding, both at saturating conditions and equimolar concentrations, is given by Equation 2.7 = 100* A/(100-A)
A = Percent inhibition caused by a sulfonamide competitor as described by Equation 2.6.

[3] It is possible that IAC inhibition was less pronounced because it did not reach equilibrium as did the ELISA system. To clarify this, an extra experiment was performed to allow the reaction mixture to be exposed to the IAC gel under conditions closer to equilibrium. The NP-sulfonamide versus parent sulfonamide competitions were mixed gently with the appropriate IAC gel in a vial for 60 min, before applying the contents to a column for normal washing and elution. These inhibition results were very similar to the corresponding flow through replicates (Table 4.2), indicating binding kinetics could not explain the difference between the binding dynamics observed by ELISA and IAC.

Table 4.2. Percent inhibition[1] of parent sulfonamide binding to immunoaffinity columns by equimolar and saturating[2] competitor sulfonamide derivatives

Sulfonamide derivative	Anti-SMT IAC		Anti-STZ IAC	
	1A	1B	1C	1D
NA	52.3 ± 0.9 a	49.4 ± 0.3 a	52.9 ± 4.8 a	60.7 ± 0.9 a
NP	56.8 ± 1.3 ab 59.4[3]	51.1 ± 0.8 a	61.1 ± 1.1 a 64.5[3]	66.5 ± 1.1 b
Succinyl	52.7 ± 0.6 a	50.0 ± 1.3 a	56.8 ± 1.7 a	67.2 ± 0.5 b
NAHis	58.8 ± 1.9 b	61.0 ± 1.6 b	56.2 ± 5.6 a	71.8 (singlet) c

[1]Percent inhibition caused by N^4-acetyl (NA), N^4-propionyl (NP), N^4-succinyl (succinyl) or N^4(N-acetyl-L-histidine) (NAHis) derivatives of sulfamethazine (SMT) or sulfathiazole (STZ) were calculated using Equation 2.6. Numbers represent average percent inhibition values from duplicates (± standard error of the mean; n=2) run through the IAC system and analyzed by the Bratton Marshall assay.

[2] The total mixed sulfonamide mole loading was at least twice the IAC saturation capacity as determined by the parent sulfonamide alone

[3] These data refer to the results of an equilibrium test, done in singlet, where the competing sulfonamides were gently mixed with the IAC gel for 60 min in a vial, then applied again to the column under normal procedures of washing and elution.

[a-c] For a given IAC, means with the same letter in a column are not significantly different ($p>0.05$).

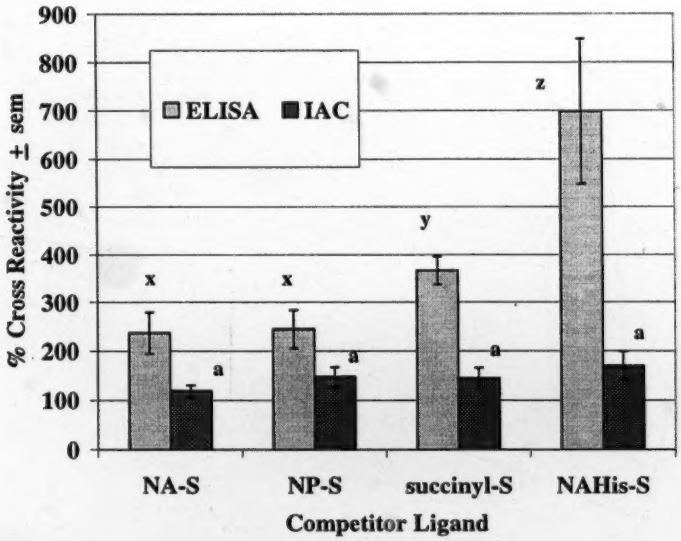

Figure 4.2. Average immunopurifed antibody cross-reactivity to competitor ligands relative to parent sulfonamide in ELISA vs IAC

Bars represent the mean cross-reactivity of four antibodies (1A, 1B, 1C, and 1D) used in both ELISA and by immunoaffinity columns (IACs) ± standard error of the mean (sem; n=4) against N^4-acetylsulfonamide (NA-S), N^4-propionylsulfonamide (NP-S), succinylsulfonamide (succinyl-S), and N^4(N-α−acetyl-L-histidine)-sulfonamide (NAHis-S). Sulfamethazine (SMT) derivatives and sulfathiazole (STZ) derivatives were used with IACs specific to SMT and STZ, respectively.

[a, x-z] Within ELISA or IAC bars, mean % cross-reactivity that have the same letter are not significantly different ($p>0.05$).

Refer to Equations 2.7 and 2.10 in Chapter 2 for cross-reactivity formulae for IAC and ELISA Format 3, respectively.

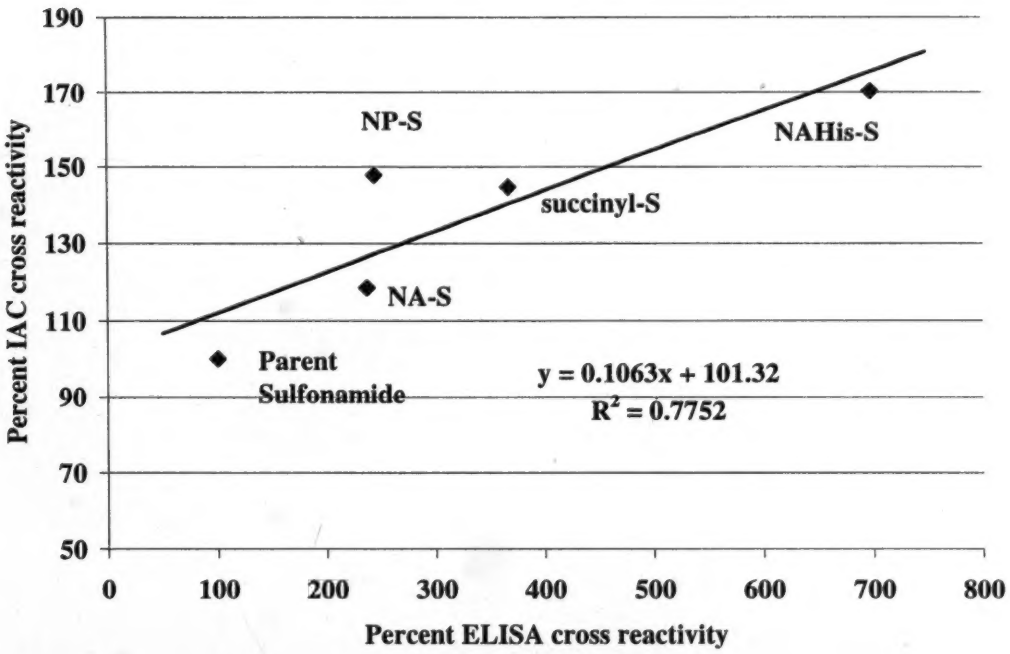

Figure 4.3. A correlation of cross-reactivity in competitive ELISA and cross-reactivity in IAC with saturation dynamics for sulfonamide derivatives

The mean cross-reactivity of four antibodies (1A, 1B, 1C, and 1D) used in both ELISA and by immunoaffinity columns (IACs) ± standard error of the mean (sem; n=4) against N^4-acetylsulfonamide (NA-S), N^4-propionylsulfonamide (NP-S), succinylsulfonamide (succinyl-S), and N^4(N-α−acetyl-L-histidine)-sulfonamide (NAHis-S). Sulfamethazine (SMT) derivatives and sulfathiazole (STZ) derivatives were used with IACs specific to SMT and STZ, respectively.

Refer to Equations 2.7 and 2.10 in Chapter 2 for cross-reactivity formulae for IAC and ELISA Format 3, respectively.

IAC could be used for sulfonamide recovery of the derivatives tested here prior to their detection. The small linker arm effects of these IACs did not grossly bias their recoveries relative to the parent sulfonamides. However, in an ideal quantitative system, the main compounds of interest and internal standard are recovered equivalently. If an IAC were to be used as a clean-up step prior to quantitative analysis, then the linker arm effect of some IACs would interfere and cause unequal recoveries under saturation conditions. Anti-SMT IAC "1B" was the only IAC tested that did not differentiate SMT, NA-SMT (the SMT metabolite), or NP-SMT (a potential internal standard for MALDI-TOF MS), and for this reason was considered a good candidate for sulfonamide clean-up of environmental samples prior to MALDI-TOF MS analysis.

4.2.3. IAC Experiment 3: Non-competitive IAC Capacity for Sulfonamide Analogues and Derivatives

IAC experiment in section 4.2.1 demonstrated a remarkable ability of immunopurified anti-SMT antibodies in IAC to recover structurally different sulfonamide compounds under non-competitive conditions. Yet also noted was its strong bias toward binding sulfonamides similar in structure to the immunogen's hapten epitope with competitive ELISA (Chapter 3, section 3.3.1) or with competitive, saturating IAC (Chapter 4, section 4.2.1). The objective of this experiment was to determine if an anti-SMT IAC would have adequate non-competitive capacity to be used practically for sample clean-up of a variety of related sulfonamides in a mixture. If there were a single, main mechanism of hapten binding conferred by the immunopurified polyclonal antibody population, or if the mechanisms of binding were equivalent with respect to their effective affinities to a given hapten, then the IAC would have similar non-competitive saturating capacities for all sulfonamides capable of binding at non-saturating levels, assuming the affinities were high enough to retain the sulfonamide.

In this experiment, the same 0.4 mL IAC possessing IA immunopurified anti-SMT antibodies was used to determine the capacity, in duplicate, for SMT, NA-SMT, NP-SMT, succinyl-SMT, SF, SMR, SDA, SPD, SDM, STZ, and NAHis-SMT

sulfonamides[1]. The development work prior to this experiment established conditions necessary for the full elution of sulfonamide derivatives from IAC "1A" (ethanol added and 10 mM HCl mixed 1:4 v/v).

Except for sulfanilamide, IAC 1A had measurable non-competitive capacities for all the sulfonamide analogues and derivatives relative to the parent sulfonamide, SMT (Figure 4.4). As shown in Figure 4.5, the capacity for a sulfonamide on the anti-SMT IAC was a function of the corresponding ELISA cross-reactivity relative to SMT (determined earlier in section 3.3.3). This linear function could be expressed by the equation; $y = 0.0633x - 4.41$, where y = log % cross-reactivity in ELISA relative to SMT, and x = % IAC capacity relative to SMT ($R^2 = 0.9328$). This correlation can predict sulfonamide capacity on an IAC for sulfonamides that have lower affinity for the antibody than the parent sulfonamide. It predicts that if a sulfonamide's cross-reactivity in competitive ELISA is less than 10^{-5} relative to the parent sulfonamide, it will just have measurable capacity on the equivalent IAC (as confirmed by sulfanilamide's almost insignificant capacity). It also predicts that if a sulfonamide's cross-reactivity in competitive ELISA is greater than 1/1000 relative to the parent sulfonamide; it will have a capacity on the equivalent IAC equal to or greater than 50% relative to SMT (as confirmed by STZ, SDA, SPD, SDM, and SMR).

The reason for the correlation between ELISA cross-reactivity and IAC capacity is not directly obvious. The mechanisms that cause a sulfonamide to compete in ELISA may not be the same processes involved in determining the capacity of a sulfonamide on the corresponding IAC. Polyclonal antibodies of 1A serum represent a heterogeneous population of affinities for sulfonamide hapten. It is likely that sub-populations of antibodies on IAC 1A have different relative affinities to the various sulfonamides tested, and the pattern of IAC binding capacities can be attributed, at least in part, to this phenomenon[2].

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[1] Sulfonamides with free N^1-amino functional groups were quantified using the BM assay, whereas the acyl derivatives (NA-SMT, NP-SMT and succinyl-SMT) were hydrolyzed first in base before analysis with the BM assay. The distinctive yellow-red colour of NAHis-SMT allowed for its direct colorimetric quantification at A_{436} using Equation 2.5a.

[2] As Muldoon et al. (2000) observed in characterizing monoclonal antibody specificity toward sulfonamides, for a given immunization of an animal, there develop a variety of molecular themes within the antibody population, each conferring different degrees of specificity to a selection of related

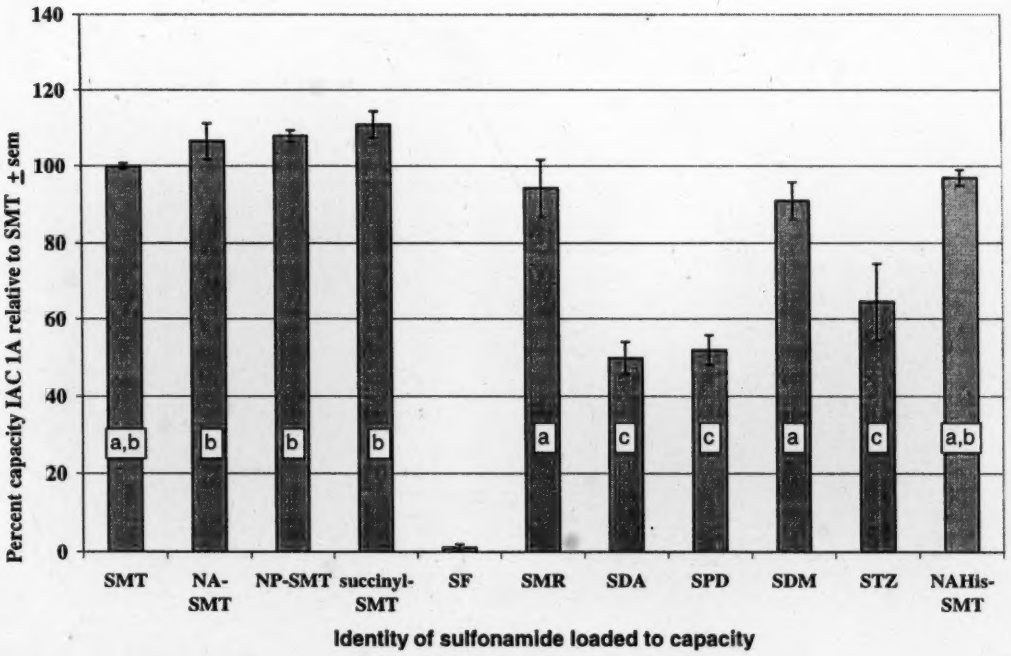

Figure 4.4. Antibody 1A IAC non-competitive capacity for several derivatives and related sulfonamides.
One 0.4 mL column was used for all tests, and had a capacity of 77 nmol for sulfamethazine (SMT). Each sulfonamide was tested on the IAC separately, and added at a loading was at least twice the IAC saturation capacity as determined by the parent sulfonamide, SMT.

Mean percent capacities were also reported (n=2 ± standard error of the mean (sem)) for N^4-acetylsulfamethazine (NA-SMT), N^4-propionyl sulfamethazine (NP-SMT), succinylsulfamethazine (succinyl-SMT), sulfanilamide (SF), sulfamerazine (SMR), sulfadiazine (SDA), sulfadimethoxine (SDM), sulfathiazole (STZ), and N^4(N-α−acetyl-L-histidine)-sulfamethazine (NAHis-SMT).

[a-c] Bars with the same letters are not significantly different ($p>0.05$).
IAC capacity was determined using Equation 2.5.

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sulfonamides. There may be sub-populations of antibodies that have sulfonamide group specificities. A sub-population that is capable of even minor cross-reaction in ELISA would be capable of recovering sub-saturating levels of this sulfonamide (Figure 3.2). The proportion of this sub-population to the whole IAC antibodies population would determine the actual capacity of an IAC for a sulfonamide. The relationship seen in Figure 3.7 is likely the consequence of a polyclonal population distribution of antibody specificities, and is indirectly related to the similarity of a sulfonamide to the parent sulfonamide of the immunogen.

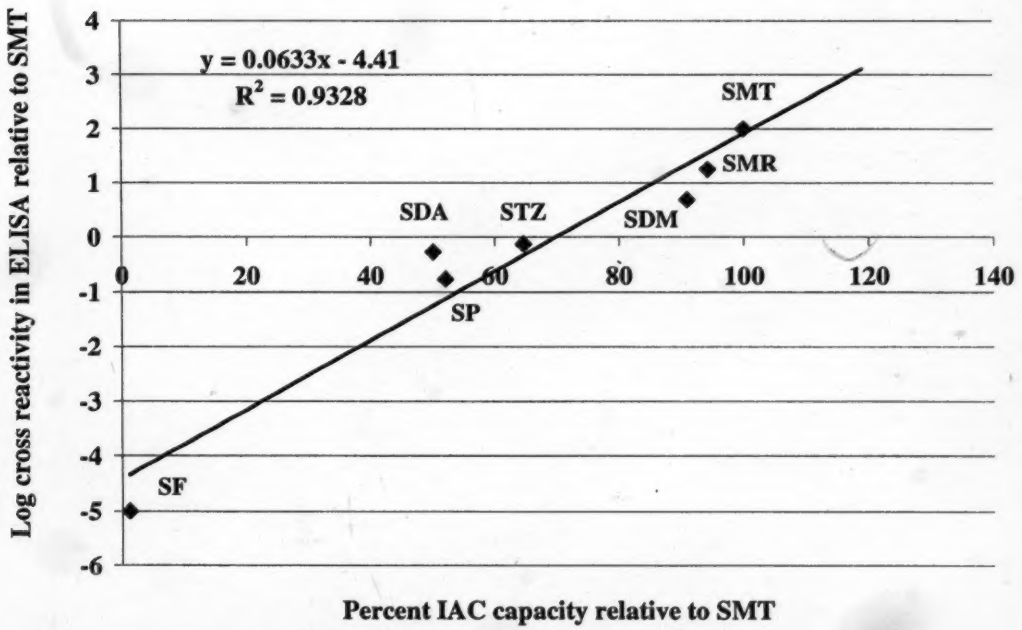

Figure 4.5. Correlation of competitor cross-reactivity in ELISA to the competitor's percent IAC non-competitive capacity relative to sulfamethazine.

ELISA cross-reactivities and IAC capacities of sulfanilamide (SF), sulfamerazine (SMR), sulfadiazine (SDA), sulfadimethoxine (SDM), sulfathiazole (STZ), sulfapyradine (SPD) were determined relative to sulfamethazine (SMT) in sections 3.3.3 and 4.2.3, respectively.

The same IAC, made with immunopurified antibodies from serum 1A, was used for all percent capacity determinations relative to SMT capacity.

In the design and development of this experiment, it was discovered that 20% ethanol was required in the 10 mM HCl elution buffer for IAC 1A to establish full sulfonamide elution in minimal buffer. Originally, 10 mM HCl pH 2.5 elution conditions had been established using IAC 1B, which were assumed to apply to all IACs made with sulfonamide-specific antibodies immunopurified using sulfonamide-hapten solid phase system and a pH 2.5 elution buffer. The fact that this was not so, makes these findings worth considering with respect to interpretation of the linker arm effects seen in the previous IAC experiments. In previous IAC Experiments 1 and 2 (see sections 4.2.1 and 4.2.2, respectively), observed linker arm effects may have been less pronounced for 1A and 1C IAC systems because the elution and regeneration buffer used (10 mM HCl, pH 2.5) did not fully elute some sulfonamide derivatives from the IAC. If IACs are not being fully regenerated, it means that the highest affinity antibodies still are bound to sulfonamide when the next experiment is conducted. This means that a sub-population of 1A antibodies with lower affinity to the sulfonamide derivatives is being used in an experiment where regeneration is not complete. This may explain why the linker arm effect was not as pronounced as one may have anticipated in the IAC Experiment 1 (section 4.2.1), because under 10 mM HCl elution protocol the IAC antibodies with higher affinity to the sulfonamide derivatives were effectively removed from the competition. If the IACs had been fully regenerated, the function established between the IAC and ELISA cross-reactivities would likely be steeper than that reported in Figure 4.3.

4.3. SUMMARY

An ideal purification system was described and then considered in relation to the theoretical IAC performance required for trace-level sulfonamide clean-up prior to analysis. Having theoretically established that a sufficiently small volume IAC was capable of extracting 1 μg SMT detectable by MALDI-TOF MS, experiments were conducted to develop and evaluate IACs for sulfonamides.

Both CNBr-activated Sepharose and AffiPrep Hz™ hydrazide supports were evaluated. The hydrazide support system had a problem with non-specific binding of sulfonamide to the support matrix, so was not acceptable for use in sulfonamide IAC.

The anti-SMT IAC alone was unlikely to achieve the sulfonamide concentration and purification goals in a one step process, so to avoid IAC "bleed", "break-out", or collapse problems, a primary, non-specific SPE concentration step would be necessary before an IAC purification step.

Anti-hapten immunoaffinity chromatography is common in commercial and research laboratories, and monoclonal antibodies are usually used for the more popular IAC procedures. If polyclonal antibodies are to be used in IAC with the same hapten capacity as monoclonal antibodies, the specific antibody fraction is required. However, polyclonal antibodies are not usually immunopurified due to problems encountered with their high affinity to the haptens and their linker attachments. Reports of immunopurification of hapten-specific polyclonal antibodies are rare, and very poor yields of 0.2% of the IgG fraction has been reported (Assil et al., 1992b)[1]. Others have found solutions by using special hapten-selection strategies during antibody immunopurification to decrease its affinity for the hapten-solid phase so as not to irreversibly bind the antibodies (Ben Rejeb et al., 1998b; Choi et al., 1997). It seems peculiar that this current research may be the only report of a successful hapten IAC enrichment of polyclonal antibodies using the same immobilized hapten for antibody purification as was used for immunization. As discussed in this chapter, the success of this antibody purification by hapten-IAC may be due, in part, to the unique acid-base chemistry of the sulfonamide molecule, the nature of which is rare amongst other haptens. This could explain the lack of literature on this type of procedure, and if this is so, then this immunopurification system may be unique to sulfonamides, or applicable only other haptens that have similar acid/base chemistry.

Competitive ELISA of sera was shown to be capable of predicting the subsequent performance of immunopurified antibodies in IAC. Under saturation conditions, the prevention of a parent sulfonamide binding in IAC by a related sulfonamide derivative, or the non-competitive capacity of an IAC for a related sulfonamide, were both shown to be

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[1] Similar to this research, the previous work by Assil et al. (1992) used sulfonamides conjugated with an azo linkage for both conjugates used as immunogens and for the IAC-purification of antibodies. As found for sulfonamide-azo-conjugate immunogens in chapter 3, strong linker arm effects would have made their antibodies possess much higher affinity to the hapten conjugates than for the free haptens. This was likely the reason they found very poor antibody recovery from their IAC when they tried to use high concentrations of sulfonamide hapten to competitively elute antibodies.

a function of ELISA cross-reactivity to the reference parent sulfonamide. This research has demonstrated the potential utility of ELISA as a screening tool in choosing polyclonal antibody sera for IAC candidates, and for predicting the subsequent IAC recovery and capacity performance. Under non-saturating conditions, anti-SMT IAC could accommodate sulfonamide compounds that were structurally different, making this IAC system capable of being a clean-up system for group specific sulfonamides. Under saturating conditions, the IACs were shown to be more discriminating among structurally related sulfonamides, and only the most similar of sulfonamides would be recovered under these competitive conditions[1]. Even under saturating conditions, the SMT derivatives, representing a metabolite and an analytical internal standard (NA-SMT and NP-SMT, respectively), were recovered similarly using SMT in IAC "1B". For this reason, this particular antibody was selected for future clean-up of SMT from environmental samples. The IACs made with this antibody were shown to be very robust; in one case, a particular column was used to extract more than 100 samples, involving future experiments with buffers and environmental samples, and did not show noticeable loss of sulfonamide capacity.

Knowledge reported in this chapter about the performance, operational ranges, and limitations of the IAC system for CNBr-activated Sepharose support, was the foundation for the solid phase immunoextraction (SPIE) system developed to detect trace-level sulfonamides as reported in Chapters 4 and 5.

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[1] The linker arm effect seen in ELISA from chapter 3 was much less pronounced in the IAC experiments with sulfonamide derivatives. This was fact was partially explainable by incomplete regeneration of some of the IACs, but this disparity was more likely due to the greater responsiveness of the ELISA measurement system.

Chapter 5

DEVELOPMENT OF SOLID PHASE IMMUNOEXTRACTION (SPIE) WITH MALDI-TOF MS FOR DETECTION OF SULFAMETHAZINE RESIDUES IN FORTIFIED ENVIRONMENTAL SAMPLES

5.1. INTRODUCTION

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is capable of detecting many antibacterial compounds simultaneously and with high sensitivity. Sulfamethazine (SMT) standards have been analyzed directly to a limit of 200 ng/L in methanol without concentration (Ling et al., 1998). Unlike other types of mass spectrometry that require extensive purification by methods such as high performance liquid chromatography (HPLC) prior to analysis, MALDI-TOF MS is relatively tolerant of sample matrix contaminants (Shaler et al., 1996; Sporns and Wang, 1998). Solid phase extraction is often employed before MALDI-TOF MS as a quick and non-specific clean-up step to enrich and purify drugs from water (Cohen and Gusev, 2002), however, drug residue analysis in complex biological samples requires more selective purification.

The goal of the research in this chapter was to use solid phase immuno-extraction (SPIE) coupled with MALDI-TOF MS to rapidly recover and detect trace levels of sulfamethazine (SMT) and its N^4-acetyl metabolite (NA-SMT) fortified in water, and from soil or manure suspensions. SMT was chosen as a drug model to examine the potential of SPIE-MALDI TOF, because SMT has been used extensively in animal agriculture for decades, and it is possible that this compound may be detected in ecosystems associated with intensive agriculture. A protocol was developed that could be used for a variety of agriculture-related environmental samples, such as water samples with unknown amounts of dissolved material, manure wastes from animals, or soil samples.

The potential of immunoaffinity chromatography for enrichment prior to MALDI-TOF MS analysis has been explored (Driedger and Sporns, 2001a; Kiernan et al., 2002; Liang et al., 1998; Neubert et al., 2002), yet none have used immuno-purification of small haptens (MW < 500 Da) like sulfamethazine in conjunction with MALDI-TOF MS. By making a high-capacity, hapten-specific immunoaffinity column (IAC), and combining this with the fast, automatable, and simple analytical features of MALDI-TOF MS, it was thought that an efficient method could be developed for the definitive detection of trace levels of drugs and their metabolites in complex environmental samples.

5.2. BACKGROUND AND METHODS DEVELOPMENT FOR SPIE MALDI-TOF MS

Solid phase immunoextraction of each environmental sample was comprised of three stages; first, concentration with HLB Plus™ solid phase extraction (SPE) cartridge, then immunoaffinity chromatography with an anti-SMT IAC, and lastly, concentration using a micro-HLB column before MALDI-TOF MS analysis (Figure 5.1). The development and rationale of each of these stages is briefly described, followed by a description of their coordinated application.

5.2.1. Initial Solid Phase Concentration

Despite the excellent binding capacity and recoveries for the immunoaffinity column (IAC) under low sample volume conditions (2-15 mL), the initial attempts to use an IAC alone to concentrate ng/L-levels of sulfonamides from water were not successful (data not presented). The large sample volumes required to collect enough analyte for MALDI-TOF MS analysis (about 1000 mL) exceeded the breakout volume of the IAC (between 100-200 mL). This breakout phenomenon is one limitation in determining the practical limits of detection in a residue analysis. Although low concentrations of herbicides in 1 L water have been extracted immunoaffinity chromatography without significant breakout (Stevenson, 2000), this is a function of the particular antiserum and its binding constant with the hapten. A preliminary concentrating step was necessary before immunoaffinity purification of SMT. Commercial Sep Pak™ C_{18} cartridges (Waters Corp., Milford, MA) are most commonly used for this purpose in residue analysis, but these exhibited breakout phenomenon at about 200 mL of water similar to the observations with the IAC. Instead, HLB Plus™ cartridges were used, which were superior in total capacity, resistant to breakout, and maintained performance even if they ran dry during use.

Although initial methods development showed the HLB Plus SPE cartridge was adequate by itself for concentration of SMT in water (data not reported), the cartridges alone were unable to provide enough purification for MALDI-TOF MS detection of SMT in complex biological suspensions such as manure or soil. When immunoaffinity chromatography was used in conjunction with HLB Plus cartridges, ng/L-levels of

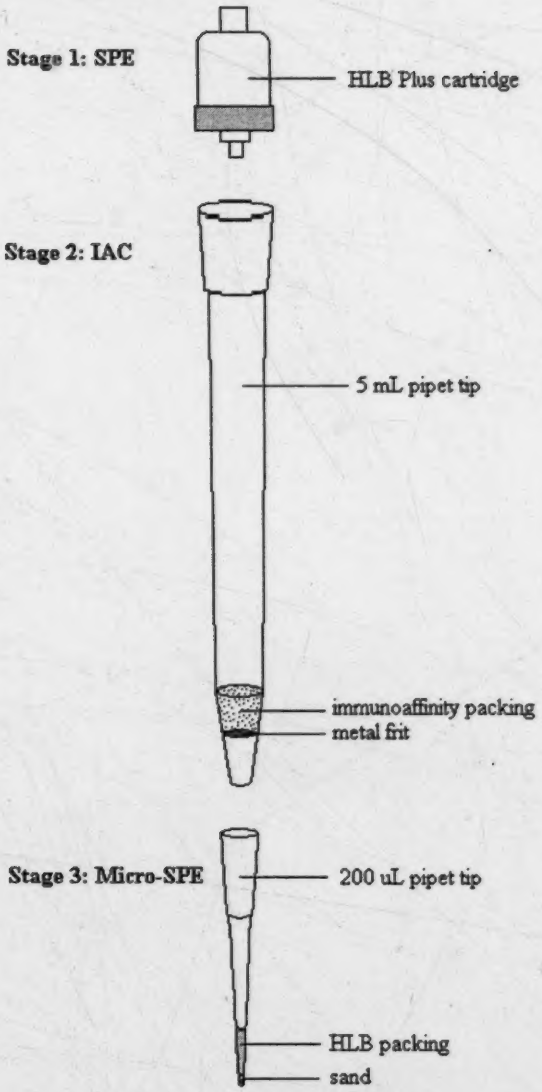

Figure 5.1. Schematic diagram of the SPIE system showing the three stages of sulfonamide enrichment achieved by solid phase extraction (SPE), anti-sulfamethazine specific immunoaffinity chromatography (IAC) and the micro-SPE column. The elution fraction of stage 1 is added discontinuously to stage 2 after dilution of the ethanolic elution solvent with PBS. The acidic elution fraction of stage 2 can be added directly to stage 3 for final concentration of the sulfonamides.

sulfonamides were purified from both water and complex biological suspensions using a batch process, and thus fulfill the objectives. In practice, using the Bruker Proflex™ III MALDI-TOF MS instrument required on-probe sulfonamide concentrations of purified sulfonamide in the low-μg/L range (1-25 μg/L) to generate substantial and unequivocal responses.

5.2.2. Purification by Immunoaffinity Chromatography

The yield of specific antibody from rabbits with the highest titres (batch 1 rabbits) represented about 14 functional reusable IAC systems per rabbit (assuming 0.4 mL IAC volume with an IAC antibody incorporation rate of 10 mg antibody/mL gel). Rabbit antibodies were immunopurified from sera and used for the immunoextraction of sulfonamides in all MALDI-TOF MS experiments applied to environmental samples. These IACs were made using anti-SMT antibodies purified from a rabbit 1B, immunized with SMT-azo-LPH, and then covalently attached to CNBr-activated Sepharose 4B. The development and the performance of anti-sulfonamide columns are reported and discussed in Chapters 3 and 4. The IACs Sepharose gel volumes were all approximately 0.4 mL, and had a capacity of 26 μg SMT/ mL gel. The IACs' used in the applied experiments with environmental samples were regenerated and reused as many as 100 times, and antibody bleed from the column was not an apparent problem in diminishing IAC capacity.

5.2.3. Final Solid Phase Extraction Concentration

Commercial micro C_{18} solid phase extraction columns (Zip Tip, Millipore, Bedford, MA) were tested to concentrate the IAC eluants further. Similar to investigations into the initial concentration of dilute solutions of SMT in large water samples, C_{18} had little binding capacity for the relatively large 4 mL volumes put through the micro columns. Consequently, micro-solid phase extraction columns were made using HLB Plus column packing material. These micro-HLB columns performed well, both in concentrating the IAC eluant and in changing the buffer to ethanol, a solvent that could be easily concentrated further and supported the dissolution of the dihydroxybenzoic acid (DHB) matrix used for MALDI-TOF MS. The mass of solid

phase sorbent material used in these columns was approximately 5 mg with total SMT capacity of about 20 μg, more than enough to handle the capacity of the IAC.

5.2.4. Coordination of All Three Stages of SPIE

The implementation of three stages of SPIE is shown in Figure 5.2, and photographs of the various columns are shown in Figure 5.3. Using a vacuum manifold 1 L samples were applied to HLB Plus cartridges in 30-45 minutes. The methanol eluants could not be applied directly to IAC because this solvent would not allow for antibody-hapten binding. However, a 3 mL methanol eluant from a HLB Plus cartridge could be easily diluted to approximately 15 mL with PBS, and then applied to an IAC. The IAC sample addition and washing were assisted by vacuum, and the sulfonamides were then eluted with 4 mL of 10 mM HCl (pH 2.5) by gravity. The 4 mL IAC eluants were rapidly applied to HLB micro columns by vacuum filtration. Sulfonamides were eluted with 25 μL of ethanol, and the eluants were evaporated to dryness in microvials and resuspended in matrix solution (10 mg DHB per mL 50% ethanol). The manual coordination of these stages allowed for 8 samples to be processed simultaneously in about 1.5 h.

5.2.5. MALDI-TOF MS Analysis

Selection of Conditions

Laser strength and detector voltage were selected to obtain optimal signal-to-noise ratios and high resolution. From the average of three spectra (each spectra representing a MALDI-TOF MS sample analysis replicate, and the sum of the response from 100 laser ionization events) the MALDI-TOF MS response for an analyte was determined as the sum of peak heights from proton, sodium, and potassium adducts together with any fragments. Peaks associated with an analyte were only used for the response calculation if they were 3 times greater than the general background noise in the spectral region of interest. Of the ionization matrices tested (2,5-dihydroxybenzoic acid (DHB), 2',4',6'-trihydroxyacetophenone, and α-cyano-4-hydroxycinnamic acid), DHB matrix was

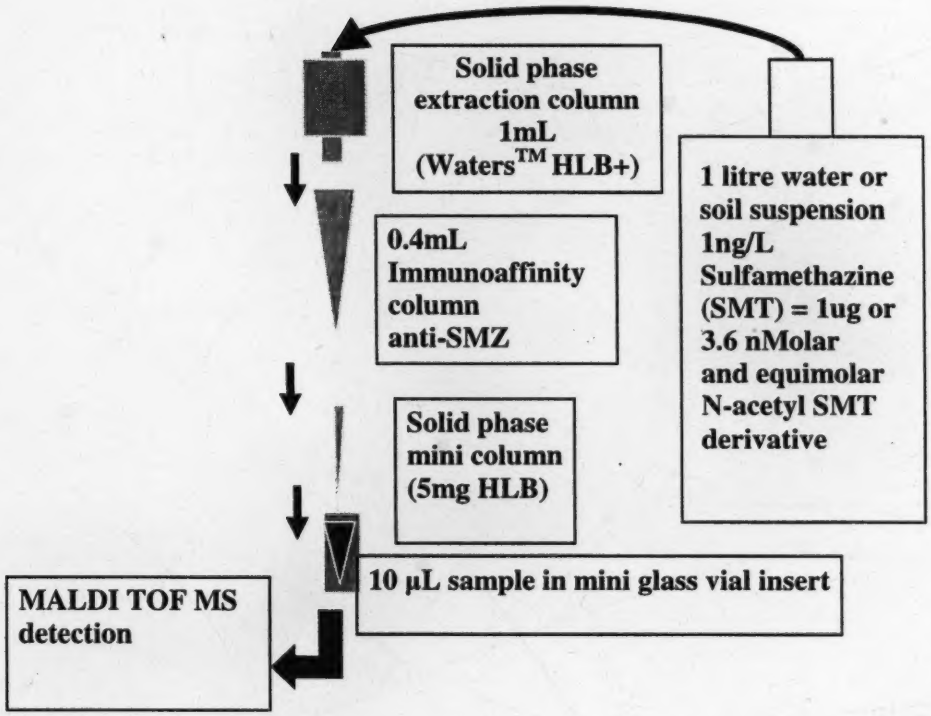
Solid phase extraction column 1mL (Waters™ HLB+)
1 litre water or soil suspension 1ng/L Sulfamethazine (SMT) = 1ug or 3.6 nMolar and equimolar N-acetyl SMT derivative
0.4mL Immunoaffinity column anti-SMZ
Solid phase mini column (5mg HLB)
10 µL sample in mini glass vial insert
MALDI TOF MS detection

Figure 5.2. A schematic diagram of the purification and concentration SPIE process of SMT and NA-SMT from a 1 L solution.

Figure 5.3. Photographs of vacuum filtration of 1L samples through HLB Plus cartridges (photograph at top), immunoaffinity column extraction system (photograph at lower right), and various commercial cartridges and home-made micro-SPE columns (photograph at lower left; Water's HLB Plus and C18 Plus Cartridges at left end, upper and lower, respectively; Water's C18 Sep Pac, 2nd from left on top; Millipore's Zip Tips, the two on the top right; and three home-made cartridges using HLB Plus material in 200 μL pipet tips, on the lower row, starting from the right).

selected because it gave substantially better signal-to-noise ratios and resolution, and resulted in no significant interferences with sulfonamide peaks.

MALDI-TOF MS Analysis of Standards

Although the objective of this research was to detect trace levels of SMT and NA-SMT, the internal standard, sulfamerazine (SMR) added to the immunoaffinity column eluants, allowed a rudimentary quantification of SMT and NA-SMT based on their relative MALDI-TOF MS responses to SMR. SMR is a good internal standard for a few reasons: (1) it is structurally very similar to SMT so it is extracted similarly to other sulfonamides in HLB columns; (2) it responds in MALDI-TOF MS similarly to SMT; (3) and it is not commonly used in animal husbandry. As shown in Chapter 4, SMR did not bind as well as SMT or NA-SMT to the anti-SMT IAC under competitive conditions, making the relative recovery of this internal standard a function of the IAC saturation state. For this reason, SMR was not an ideal internal standard, yet could be added at the end to determine sulfonamide recoveries from fortified samples based on relative MALDI-TOF MS responses to SMR using Equation 2.13 as described in the experimental methods. No internal standard was added at the beginning of the SPIE process, so proper quantitative estimates could not be made using the SMR internal standard. For quantitative SPIE MALDI-TOF MS of sulfonamides an internal standard added at the beginning of the SPIE process would minimize the high sample-to-sample variability associated with multi-step extractions (an internal standard capable of being added at the beginning of SPIE is considered in Chapter 6 in analysis of farm samples).

SMR and SMT had similar MALDI-TOF MS responses, yet the response of NA-SMT was less than half of SMT (0.99 ± 0.12 and 0.42 ± 0.06 response ratios for SMT/SMR and NA-SMT/SMR, respectively). This finding, together with the variability associated with well-documented inconsistencies of analyte/matrix co-crystallization, emphasize the need for accurate determination of response factors, the acquisition of replicate spectra, and consideration of several other important MALDI-TOF MS variables and when attempting quantification using MALDI-TOF MS (Cohen and Gusev, 2002). MALDI-TOF MS peaks for DHB matrix alone did not conflict with theoretical isotopic molecular weight/charge ratio (*m/z*) for SMT, NA-SMT, or SMR, except for very minor peaks at *m/z* 215 and 317 (see Table 5.1 and Figure 5.4).

Table 5.1. Isotopic molecular weights for sulfonamide parent molecules (M), cationic adducts, and fragment ions.

	M	$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	$[M-SO_2+H]^+$
sulfamethazine (SMT) ($C_{12}H_{14}O_2N_4S$)	278.1	279.1	301.1	317.1	215.1
N^4-acetylsulfamethazine (NA-SMT) ($C_{14}H_{16}O_3N_4S$)	320.1	321.1	343.1	359.1	257.1
Sulfamerazine (SMR) ($C_{11}H_{12}O_2N_4S$)	264.1	265.1	287.1	303.0	201.1

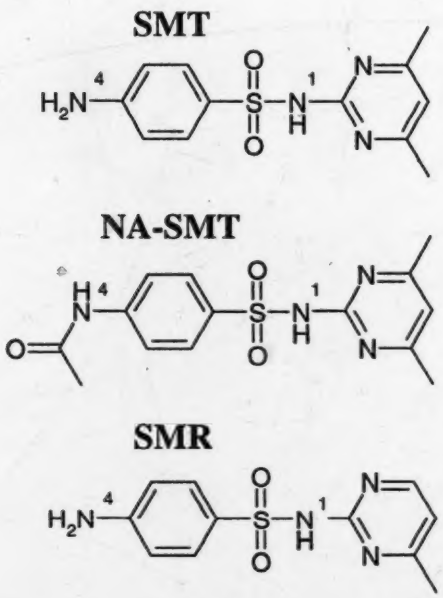

Figure 5.4. Chemical structures of sulfamethazine (SMT) , N^4-acetylsulfamethazine (NA-SMT) , and sulfamerazine (SMR).

Controls that excluded SMR showed no spectral peaks typical of SMR, whereas SMR peaks were easily seen in the spectra from samples including internal standard but not fortified with SMT or NA-SMT.

5.3. SPIE MALDI-TOF MS RECOVERY AND DETECTION OF SMT AND NA-SMT FORTIFIED IN ENVIRONMENTAL SAMPLE MATRICES

5.3.1. Testing of Unfortified Samples

Unfortified samples were first tested by SPIE MALDI-TOF MS to determine their sulfonamide status, then these samples could be fortified and tested again to evaluate SPIE MALDI-TOF for sulfonamide recovery and detection performances. With one exception, water, soil, and manure samples were taken from non-agricultural sources that would have little chance of having sulfonamide residues. These include highly purified laboratory water, a soil sample (soil 1) from the river bank of the Saskatchewan River near the University of Alberta, and composted pig manure from the University of Alberta Swine Unit from animals that had no history of sulfonamide administration. The one soil sample (soil 2), which had a possibility of containing sulfonamide, was collected from a University Farm site at Ellerslie, south of Edmonton. Regardless, it was not expected to find sulfonamides in this sample because this site did not a history of spreading animal manure since 20 years previously. Then all samples were tested at various sulfonamide fortification levels (0, 0.1, and 1.0 ng/L levels simultaneously for both SMT and NA-SMT) to determine if sulfonamides could be recovered and detected using MALDI-TOF MS.

No peaks associated with SMT and NA-SMT were identified for the unfortified water, soil 1, and manure samples, but small but significant peaks were consistently observed in the unfortified soil 2 sample, close to the limit of detection (Figure 5.5). These peaks at *m/z* 321.1 and 343.1, representing the $[M+H]^+$ and $[M+Na]^+$ adducts of NA-SMZ, respectively, corresponded to roughly 2 ng/L on a dry weight basis, when calculated using the same rate of recovery as observed for NA-SMT in fortified soil 2 samples. It was established that manure of unknown origin was spread at the site where soil 2 was collected approximately 20 years ago, so it is possible that the manure

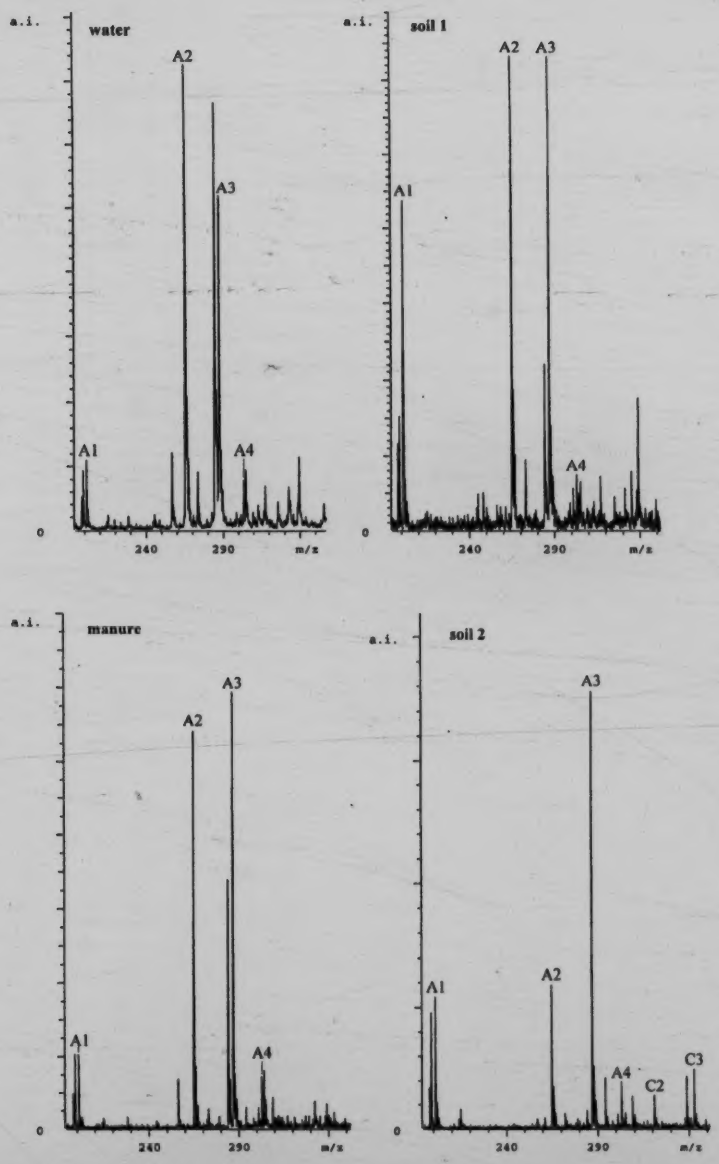

Figure 5.5. Typical MALDI-TOF MS spectra of unfortified samples; water, and suspensions of soil 1, soil 2, and composted manure, processed through the SPIE system, with added SMR. Letters A and C represent peaks associated with SMR and NA-SMT, respectively. The numbers 1, 2, 3, and 4 following the letter represent sulfonamide fragment and proton, sodium, and potassium adducts, respectively. Peak C represents the potential NA-SMT residue or contaminant of this unfortified soil sample. No peaks associated with sulfamethazine were observed. Three spectra were averaged from each replicate sample to determine sulfonamide levels, and at least two SPIE MALDI-TOF MS determinations were made for each sample. Ordinate scale is an arbitrary intensity (a.i.).

contained NA-SMT residues that did not leach from the soil. It is unlikely that laboratory contamination can explain the presence of these peaks, because they appeared in no other unfortified samples analyzed. Also, SMT and NA-SMT were always added in equal mass ratios, and SMT inherently has more than a two-fold better response relative to NA-SMT, so it seems unlikely that NA-SMT would appear as an isolated accidental contaminant. Repetition of sampling and analysis with other validated methods would be required to make any conclusions about the presence of NA-SMT residues in this soil. These anomalous signals exemplify the difficulty in trace residue analysis to establish adequate negative controls. When residue analysis approaches sub-ng/L levels of detection, it is difficult to define a sample matrix that can be guaranteed to not contain measurable quantities of analyte. False positives become a problem due to laboratory or sampling contamination, and so reliable negative controls are necessary. However, positive identification must rely upon proper sampling and laboratory practices to avoid sample contamination, and be combined with definitive analytical techniques that are not prone to multiple interpretations.

5.3.2. Detection of SMT and NA-SMT in Fortified Samples

The water, soils, and composted manure samples, fortified with 3.60 nmol of both SMT and NA-SMR, showed strong peaks associated with the fortified sulfonamides and the internal standard (Figure 5.6). SMT and NA-SMT concentrations in fortified water samples were 1.00 ng/L and 1.15 ng/L, respectively. When taking the moistures of the soils and manure into consideration, these sample slurries were marginally less concentrated by aqueous volume than the corresponding water samples. The concentrations on a dry weight basis of sulfonamides detected in soils 1 and 2 and composted manure samples, were 12.2, 11.9, and 14.5 ng/L, respectively, for SMT, and 14.0, 13.7, and 16.6 ng/L, respectively, for NA-SMT. These initial concentrations, when processed through the system, produced very strong signals in the mass spectra. Based on this high response and relatively low noise, detection of concentrations nearly 10-fold less would be possible for this system. Water samples fortified with 0.360 nmol of both of SMT and NA-SMT (0.100 ng/L and 0.115 ng/L, respectively) (Figure 5.7), showed significant SMT and NA-SMT signals more than three times above the average

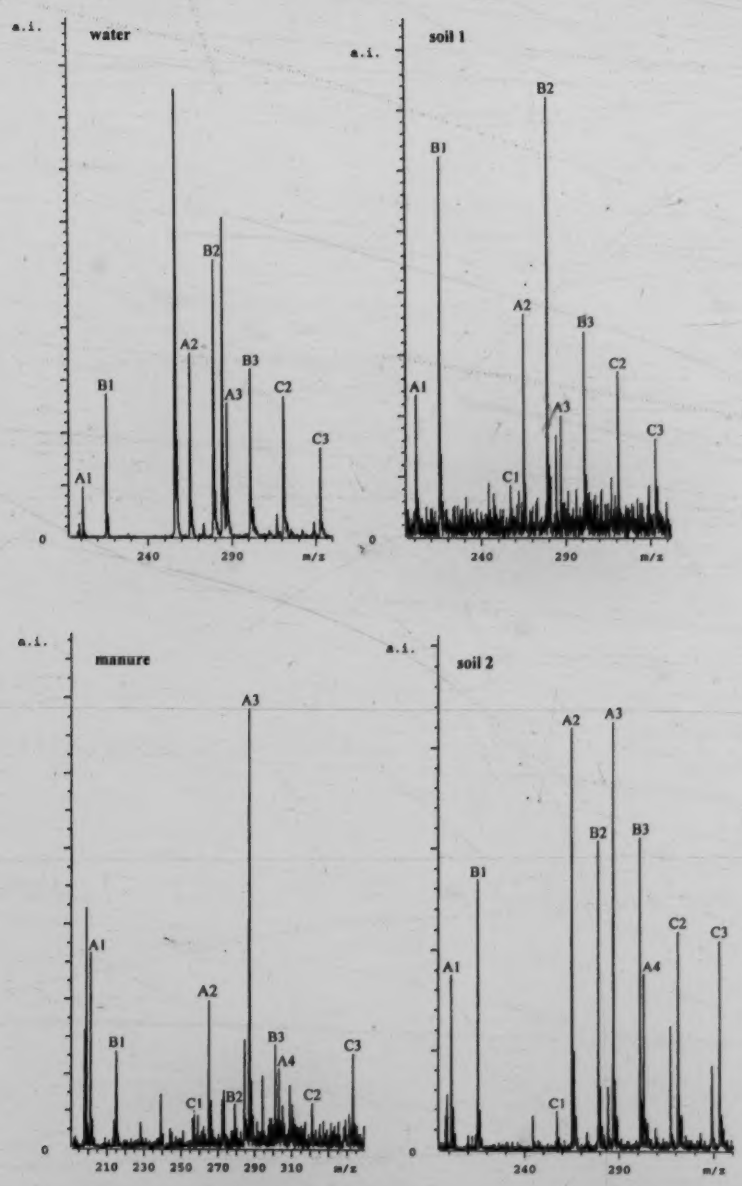

Figure 5.6. Typical MALDI-TOF MS spectra of fortified samples: 1 L water, and suspensions of soil 1, soil 2, and composted manure (1 L deionized water added to each), fortified each with 3.60 nmol of SMT and NA-SMT, processed through the SPIE system, with added SMR. Letters A, B, and C represent peaks associated with SMR, SMT, and NA-SMT, respectively. Numbers 1, 2, 3, and 4 following a letter represent sulfonamide fragment and proton, sodium, and potassium adducts, respectively. Three spectra were averaged from each replicate sample to determine sulfonamide levels, and at least two SPIE MALDI-TOF MS determinations were made for each sample. Ordinate scale is an arbitrary intensity (a.i.).

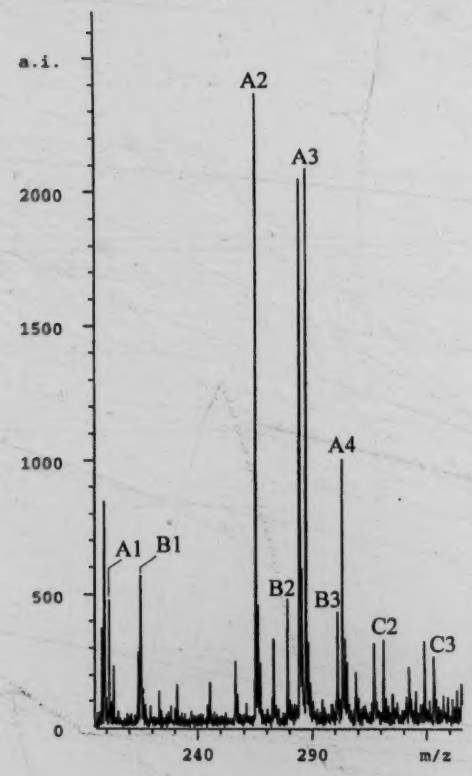

Figure 5.7. Typical MALDI-TOF MS spectrum of 1 L water fortified with 0.36 nmol each of SMT and NA-SMT, processed through the SPIE system, with added SMR. Letters A, B, and C represent peaks associated with SMR, SMT, and NA-SMT respectively. Numbers 1, 2, 3, and 4 following a letter represent sulfonamide fragment and proton, sodium, and potassium adducts, respectively. Three spectra were averaged from each replicate sample to determine sulfonamide levels, and at least two SPIE MALDI-TOF MS determinations were made for each sample. Ordinate scale is an arbitrary intensity (a.i.).

background noise in the spectral vicinity, making the sensitivity of SPIE MALDI-TOF MS comparable to the performance reported for other mass spectrometric methods in residue analysis of water (Cavaliere et al., 2003a; Lindsey et al., 2001; Pfeifer et al., 2002).

5.3.3. Recovery of SMT and NA-SMT in Fortified Samples

Recoveries of SMT and NA-SMT for fortified samples in separate stages of the SPIE system were determined colorimetrically using the Bratton-Marshall (BM) assay with buffers and volumes appropriate for each stage of SPIE (1 L water for initial SPE, 15 mL PBS with 20% ethanol for IAC, and 4 mL 10 mM HCl for micro-SPE). As shown in Table 5.2, both sulfonamides exhibited similar recovery patterns through the system. Approximately half of the sulfonamide content was lost in the first solid-phase extraction step. IAC was not selective for one sulfonamide over the other, and both were recovered from this stage at nearly 100%. There was a similar loss of approximately 20% of both sulfonamides in the final micro-extraction step. Overall, the colorimetric estimates of each stage of the SPIE system predict that SMT would be recovered at a rate of 44% through the whole system and NA-SMT would be recovered at a rate of 34%. Despite losses, the SPIE system demonstrated enough concentration effect to allow sulfonamide detection in samples with even sub-ng/L initial sulfonamide concentrations.

Recoveries of SMT and NA-SMT from water, soil, and manure systems fortified at levels shown in Table 5.3 were determined by MALDI-TOF MS with the addition of SMR as internal standard at the micro-SPE stage. SMR was only used as a recovery

Table 5.2. Percent recovery of sulfonamides from each stage of the SPIE system [1]

Stage	Description	SMT	NA-SMT
1	Solid phase extraction (SPE) from 1 L water	57.3 ± 9.1	42.7 ± 4.3
2	Immunoaffinity chromatography (IAC) from 15 mL PBS	95.3 ± 4.7	101.6 ± 1.9
3	Micro-SPE from 4 mL 10 mM HCl	80.2 ± 2.6	78.9 ± 3.5
Together	Expected total SPIE recovery[2]	44%	34%

[1] Ten nmol of each sulfonamide was fortified into 1 L water, 15 mL PBS with 20% ethanol (v/v), or 4 mL 10 mM HCl for the SPE, IAC, or micro-SPE stages, respectively. Recoveries were determined colorimetrically using the Bratton-Marshall assay. Values represent the average of 2 determinations ± standard error of the mean.

[2] Theoretical values were determined by multiplying recovery rates from stages 1-3.

Table 5.3. Percent recovery of sulfonamides from fortified environmental samples using the SPIE MALDI-TOF MS system

Sample	Spike level (nmol)[1]	Number of trials	SMT[2]	NA-SMT[2]
Water	3.6	3	44.2 ± 3.1	40.7 ± 2.9
Water	3.6	2	43.3 ± 14.5[3]	43.3 ± 14.5[3]
Water	0.90	2	39.0 ± 16.8	48.3 ± 9.2
Water	0.36	2	91.3 ± 17.4	59.5 ± 4.7
Soil 1	3.6	2	43.7 ± 4.4	37.9 ± 4.1
Soil 2	3.6	3	24.3 ± 5.1	25.5 ± 4.8
Manure	3.6	3	8.0 ± 6.3	20.0 ± 5.8

[1] Units refer to the quantity of each sulfonamide added to 1 L water, aqueous soil suspension (10% w/v), or aqueous manure suspension (10% w/v).

[2] Unless denoted otherwise, values were determined by MALDI-TOF MS.

[3] Values were determined colorimetrically by the Bratton-Marshall assay; they represent the combined recoveries of SMT and NA-SMT as the two sulfonamides could not be differentiated in this case.

standard and a positive control for MALDI-TOF MS sulfonamide response. There were problems associated with non-equivalent recovery of SMR relative to SMT and NA-SMT at the immunoaffinity chromatography stage (stage 2), so SMR could not added to the sample at the beginning of the SPIE process to be reliably used as a quantitative reference (refer to IAC Experiment 1 of Chapter 4, section 4.2.1). An average recovery rate for SMT, NA-SMT and SMR of 81 $\pm$ 0.01% (n=3) from the micro-SPE column (determined colorimetrically) was factored in (F in Equation 2.13) to estimate recoveries using MALDI-TOF MS. In cases where sulfonamides were present in complex organic mixtures (i.e. soil or manure), the recoveries were expected to decrease, causing subsequent increases in detection limits. The lower recoveries for soil 2 and manure samples were likely due to greater sulfonamide losses at the initial SPE step, but this could not be verified colorimetrically due to large sample blank interferences at an absorbance at 545 nm, the same wavelength used for the Bratton-Marshall assay. SPE cartridges are not selective like the IAC, and it is possible the HLB Plus cartridge became saturated with hydrophobic organic compounds like lipids or proteins, which likely vary in quantity and quality from sample to sample.

High recoveries were determined for the lowest concentration (0.36 nmol in 1 L) of SMT and NA-SMT in water (91.3 $\pm$ 17.4% and 59.5 $\pm$ 4.7%, respectively, n=2). Column saturation dynamics cannot explain this observance because sample loading of 0.9, 3.6, and 10 nmol all exhibited similar recovery rates (Tables 5.2 and 5.3). The recovery of this lowest sulfonamide concentration represents data that were near the detection limits of the MALDI-TOF MS method, and large spectral variability associated with these low responses are likely responsible for an over-estimation of the recovery rate. Another possible explanation to describe higher recoveries for lower IAC loading, as discussed in Chapter 4, a polyclonal population of antibodies bound to the IAC likely have different relative affinities to sulfonamides. Antibodies may be sequentially saturated by sulfonamides based on the order of their relative affinities, and thus affect breakout and recovery phenomena in stages, depending on the column loading.

5.4. SUMMARY

As an adaptable model for other drugs and haptens, this chapter has described the recovery and detection of trace levels of sulfamethazine and its N^4-acetyl metabolite fortified into water, or soil or manure suspensions, using solid phase immuno-extraction (SPIE) coupled with MALDI-TOF MS (Grant et al., 2003). Based on the combined response intensity spectral patterns of parent ions and three other adducts and fragment peaks, the presence of SMT and NA-SMT was confirmed in fortified water at sub-ng/L levels, and in soil and manure matrices at low-ng/L levels. The standard, SMR, acted as an internal positive control and enabled the determination of recovery rates. The current method is only qualitative, because the internal standard, SMR, would not be recovered in a consistent manner relative to SMT and NA-SMT (from Chapter 4 results). A different internal standard, capable of being recovered equivalently to SMT and NA-SMT by each stage of SPIE, will be required for future quantification work. An internal standard used for quantification of sulfonamides will be necessary to compensate for the large variations in SPIE recovery and in MALDI-TOF MS response.

The initial SPE extraction step was the source of low and variable SPIE recoveries, and improvements at this stage of the process would provide the greatest gains in SPIE performance. The current detection limitations are within the same order of magnitude as reported for residue analysis by other MS instruments for samples like manure (Pfeifer et al., 2002), and approximately ten times higher than MALDI ionization techniques employing quadrupole filters in water analysis (Hirsch et al., 1999). Currently, the SPIE process takes about 1.5 h per batch (8-24 samples/batch) for sample enrichment, 5 min per batch for probe preparation, and 5 min per sample to acquire and process the spectrum, representing two or three-fold improved analysis efficiency compared to other conventional and definitive methodologies.

Chapter 6

SPIE MALDI-TOF MS ANALYSIS OF SULFAMETHAZINE RESIDUES IN ENVIRONMENTAL SAMPLES FROM A PIG FARM

6.1. INTRODUCTION

Validating an analytical system first involves being able to reproducibly measure pure analyte(s) of interest, both individually and resolved from a mixture of related compounds. The system must then be able to recover and detect the analyte when it is fortified into a sample matrix where it would be found naturally. All sample matrices of interest must be tested separately. The previous chapters have established the capability of solid phase immunoextraction (SPIE) with matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to detect both sulfamethazine (SMT) and its N^4-acetyl metabolite (NA-SMT) in fortified water, soil, and manure samples. The research reported in this chapter was to further validate SPIE MALDI-TOF MS as a means of detecting low level sulfonamides incurred in the environment by normal agricultural practices, and to initiate the validation process of using it as a quantification tool.

If an analyte of interest is not detected in a sample, it is not known whether that is because it is not present in measurable quantities or if it is simply not recoverable. This dilemma exists in quantifying incurred samples because the "true" analyte concentration can never be known from fortification recovery studies. The recovery of a fortified compound may not approximate its recovery rate in a natural sample due to the different nature of an environmental sample matrix. Within the ecological metabolism of the environment, it is possible that a sulfonamide made be degraded, undergo unknown conjugation reactions, or in a pond or lake system, bind to sludge or other solid material, settle to the bottom of a waste lagoon, and be unavailable for recovery in the aqueous domain above. Nonetheless, in this project, the SMT mass used continually in pig feed at a farm and total solid and liquid inputs could be crudely estimated for one of the waste lagoons sampled. Consequently, the expected sulfonamide concentration (SMT and metabolite, NA-SMT) incurred by normal farming practices could be estimated as an approximate reference for the SPIE MALDI-TOF MS system.

6.2. FARM SAMPLING AND EXPECTED VALUES

Environmental samples were collected from an Alberta pig farm with a 23-year history of spreading manure from pigs that had been fed SMT-medicated feed. This farm

site was selected based on both the extended period of time SMT was used and also due to intensive manure spreading practiced there. Samples were selected at sites to maximize the likelihood of SMT recovery, and their testing provided a means of proving the detection capabilities of SPIE MALDI-TOF MS for both SMT and NA-SMT incurred by normal agricultural practices in manure, soil, and water. This research cannot be considered a statistically valid survey, thus the low level findings of SMT and NA-SMT in soil and field pond water here cannot be extrapolated to other farms.

At the farm, a cut-line pasture (site 1) was commonly sprayed with manure from lagoon 1, and an agricultural field used for growing grain was commonly sprayed with manure from lagoon 2. Standing pond water from two different ponds at site 1 was collected and analyzed, and two soil samples each from sites 1 and 2 were analyzed. Samples were obtained and analyzed from the pit beneath the weaner pigs in the barn, two lagoons[1] fed by the effluent of the barns, and also water from the farm well.

Information gathered from the farmer allowed an estimation of the sulfonamide levels to be expected in the waste lagoon 1, the lagoon fed by the weaner pigs and the sow barn (Figures 6.1 and 6.2).

The following general assumptions were important in making this estimation:

1. There was no total sulfonamide degradation, and the amount of SMT and NA-SMT in the lagoon waste material would be fully conserved with respect to the amount of SMT fed the animals (i.e. other metabolites are not significant).
2. Sulfonamide concentrations within the waste lagoons were uniformly distributed.
3. Sulfonamide recovery potential was 75% (as measured by SMT and NA-SMT) of the amount added to the system by feed. Losses of 25% could occur due to production of sulfonamide metabolites not detected by the SPIE MALDI-TOF MS system, or by

[1] The two lagoons were fed by the effluent from the weaner pig and sow barns (lagoon 1), and fed by the effluent of the grower barn (lagoon 2; the barn that held growing pigs destined for slaughter).

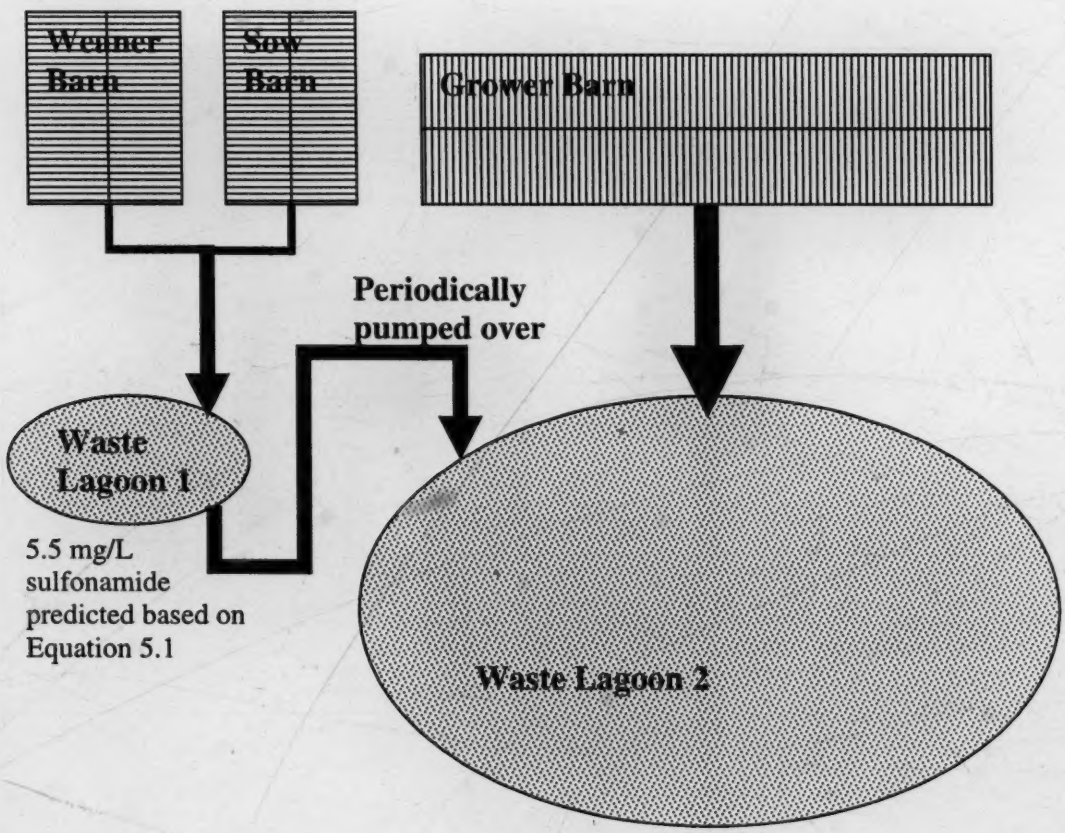

Figure 6.1. A schematic diagram showing the flow of waste materials at the pig farm from the weaner, sow, and feeder barns to lagoons 1 and 2. The areas of the barns and lagoons, and the size of the arrows representing relative waste flow rates, are not drawn to scale.

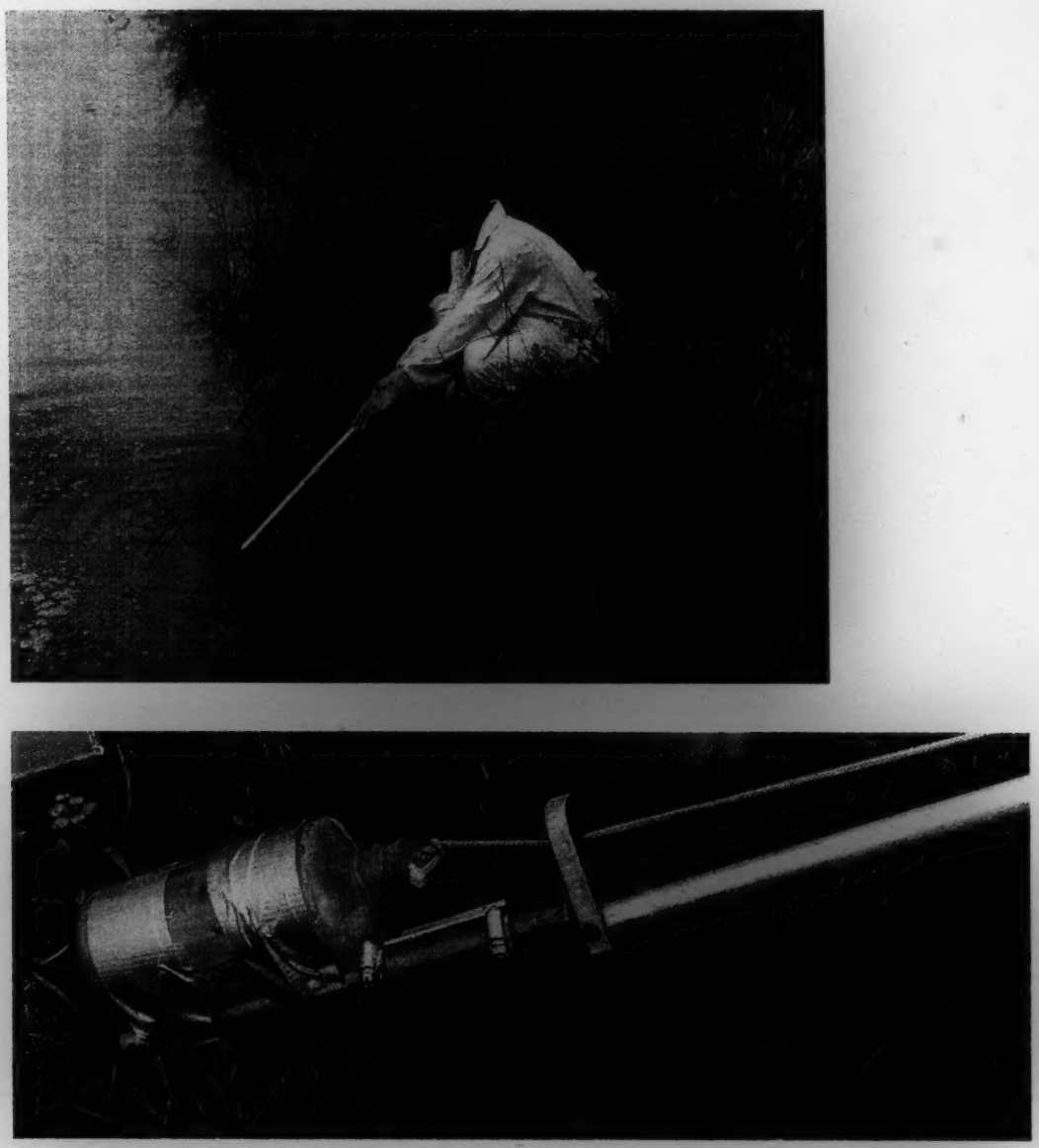

Figure 6.2. Sampling from the lagoon. In the top photograph, the author is sampling waste manure slurry from lagoon 2 at approximately 2 m depth using a bottle on a long pole. The bottom picture shows the 1 L bottle attached to the end of the pole, plugged with a cork, which can be opened for sampling with a line connected to the stopper.

binding of sulfonamides to lagoon solid materials and removing them from the aqueous fraction[1].

4. The lagoon waste material was represented by feed and water inputs to the animal barns only, and volume contributions by rain or evaporation were insignificant.

5. The sulfonamide in the feed was in the form of SMT at 220 mg/kg as reported by the feed company[2].

6. Weaner pigs were the only significant source of SMT being introduced to the environment. Periodically sows or growers would get treated with sulfonamide for an acute infection, and but routine use of sulfonamide in the feed for prevention of disease was restricted to the weaner pigs.

7. There were 135 weaner pigs eating approximately 3000 kg feed/month, and this mass is assumed to contribute an equivalent numerical volume measured in litres to lagoon 1.

8. No feed wastage estimate was made, so it is assumed to be zero.

9. 90,000 L/month of waste is contributed to lagoon 1 by a continuous flow watering system in the weaner barn (61,000 L/month), weaner barn cleaning (5,000 L/month), weaner barn feed (3,000 L/month), and an estimated total contribution from the sow barn (21,000 L/month).

Based on the general and specific assumptions, the total SMT residues expected to be measured in waste lagoon 1 were determined by Equation 6.1. Volume outputs from the grower barn were more difficult to make, so an estimate for sulfonamide concentration in lagoon 2 was not practical.

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[1] Hydroxylation metabolites and other minor sulfonamide metabolites of the pig may not be extracted by the immunoaffinity column in the SPIE process and thus not detected. Also conjugation reactions of sulfonamides to proteins in the lagoon sludge or possible sulfonamide degradation by the metabolic processes of the lagoon could decrease the sulfonamide levels in the aqueous fraction of the waste lagoon.

[2] Most pig farmers use none or 110 mg/kg sulfamethazine in the feed. The higher-than-normal sulfonamide rate given to these pigs was authorized and monitored by the farmer's "herd-health" veterinarian, as a means of treating a chronic respiratory problem in the pigs.

Equation 6.1. ***[SMT residues] estimated for waste lagoon 1*** **(mg/L) =**

([SMT in feed] µg/L or mg/kg)[1] x (weaner barn feed rate kg/month) x (recovery potential)
(Rate of water entering lagoon 1 in L/month)

= (220 µg/L) x (3000kg/month) x 0.75 = 5.5 mg/L

6.3. QUANTIFICATION AND INTERNAL STANDARDS

6.3.1. Selection of Internal Standard, NP-SMT, for Quantification

There are specific criteria established to validate an analytical system as quantitative, as described in Chapter 1. In general, a quantitative system must consistently recover an analyte fortified into a given sample matrix over a range of concentrations, and the analyte responses must be statistically reproducible relative to an internal or an external standard. A major limitation to accurate quantification with MALDI-TOF MS is the high spot-to-spot and sample-to-sample variability that results from poor crystal homogeneity and variable incorporation of analytes into the crystal bed. The absolute analyte response in the SPIE MALDI-TOF MS system used for a given sulfonamide sample sometimes varied by an order of magnitude, both because of the inherent variability of MALDI-TOF MS, and also because of the large variability in SPIE recovery rates for different sample matrices. It would be impractical to use either a direct signal response or an external standard for SPIE MALDI-TOF MS, thus an internal standard was necessary.

Both the N^4-propionyl derivative of SMT (NP-SMT) and sulfamerazine (SMR) were used as internal standards for SPIE MALDI-TOF MS analysis of the farm samples. SMR was used earlier in the analysis of environmental sample matrices fortified with SMT and NA-SMT (Chapter 4). The MALDI-TOF MS response of SMR was almost identical in magnitude compared to SMT, and its fragment and adduct peaks were well resolved from those of the matrix and other sulfonamides. SMR is not commonly used in veterinary medicine, and was a suitable MALDI-TOF MS standard because of its structural and chemical similarity to SMT. However, having one less methyl group than SMT was critical in causing SMR to have inconsistent anti-SMT-IAC recoveries

(Chapter 4). SMR was subject to variability in recoveries in the first two stages of the SPIE procedure, so could not be used as a standard for quantification. Instead SMR was added after the IAC step of the SPIE process, acting as a positive control by adjusting for the large variability in MALDI-TOF MS response, and also as a reference to determine sulfonamide SPIE recovery rates.

An internal standard was still needed to allow the quantification of SMT and NA-SMT, capable of being added to a sample before sulfonamide extraction, and then be efficiently recovered by SPIE equivalently to SMT and NA-SMT. A ^{13}C isotope of SMT (MW 284.1), which is a commercially available product used for the internal standard described by official methods for GC-MS analysis (Official Methods of Analysis of AOAC International, 2000), would have been an ideal internal standard if it did not interfere with matrix peaks. However, a major MALDI-TOF MS matrix peak at 284 *m/z* for 2,5-dihydroxybenzoic acid (DHB) would have interfered with this standard, making a change of matrix necessary. DHB was superior to other matrices tested, providing the best signal to noise ratios, controllable and reproducible fragmentation, and a lack of mass interferences with sulfonamides tested. Instead of sacrificing assay sensitivity by changing the matrix, a different internal standard was synthesized. This chapter reports the testing of NP-SMT as an internal standard, which had not been available for the initial SMT detection work reported in Chapter 5. It does not occur as a natural metabolite of SMT, and it was recovered equivalently to SMT by the anti-SMT "1B" IAC column under saturating and non-saturating conditions (Chapter 4). NP-SMT has the correct structural and chemical similarities to SMT and NA-SMT, from both immunological and MS-response perspectives. NP-SMT was added to samples as an internal standard prior to sample purification and concentration, and then SMR was added at the end of the clean-up process as a positive control and a means to determine NP-SMT recovery rate (see Figure 6.3 for chemical structures and Table 6.1 for isotopic molecular weights of sulfonamides pertinent to this experiment).

6.3.2. MALDI-TOF MS Internal Standard Response Ratios

SMT consistently had a greater response than the other two acyl derivatives, and as noted before, had a significantly larger fragment peak. NA-SMT responded very

Table 6.1. Sulfonamide adduct/fragment molecular weight chart for MALDI-TOF MS

	M	$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	Fragment $[M-SO_2+H]^+$
Sulfamethazine (SMT) ($C_{12}H_{14}O_2N_4S$)	278.1	279.1	301.1	317.1	215.1
N^4-Acetylsulfamethazine (NA-SMT) ($C_{14}H_{16}O_3N_4S$)	320.1	321.1	343.1	359.1	257.1
N^4-Propionylsulfamethazine (NP-SMT) ($C_{15}H_{18}O_3N_4S$)	334.1	335.1	357.1	373.1	271.2
Sulfamerazine (SMR) ($C_{11}H_{12}O_2N_4S$)	264.1	265.1	287.1	303.0	201.1

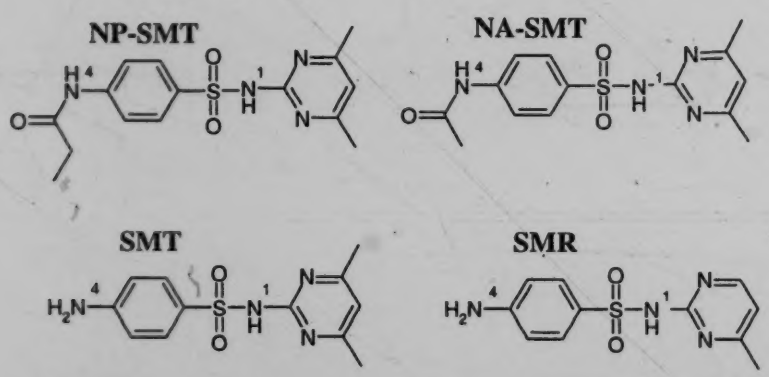

Figure 6.3. Structures of sulfamerazine (SMR), sulfamethazine (SMT), and the N^4-acetyl (NA-SMT) and N^4- propionyl (NP-SMT) derivatives of sulfamethazine.

similarly to NP-SMT in MALDI-TOF MS, which was expected because they are structurally similar. The response ratios of SMT and NA-SMT relative to NP-SMT were 2.46 and 0.93, respectively, as determined from the linear correlations of their responses over a range of sulfonamide concentrations (Figure 6.4). The mass of NP-SMT applied to the target probe was constant for the response ratio determination (0.36 nmol), and SMT and NA-SMT quantities were varied and analyzed concurrently at equivalent mole amounts greater and less than 0.36 nmol. To make the response ratios applicable and valid, it was important that the sulfonamide response ratios be determined for concentration ranges expected while analyzing trace sulfonamide residues (i.e. ng/L level). The 0.36 nmol NP-SMT value was selected as the reference on-probe standard mass, because it was equivalent to the SMT mole quantity if a 1 ng/L sample (1 L) were concentrated to 10 µL, and the normal 1 µL volume was applied to the target for analysis.

Conventionally, confirmatory MS identification of a given compound requires the minimum detection of three associated ions. In MALDI-TOF MS this requirement was fulfilled for sulfonamides due to the presence of a sulfonamide fragment and proton, sodium, and potassium adducts (Table 6.1) that had response patterns diagnostic to sulfonamides.

6.4. SULFONAMIDE ANALYSIS OF FARM SAMPLES

6.4.1. Recovery Rates of NP-SMT Standard Relative to SMR Standard

A water recovery test was performed to determine the recovery rates of the SPIE system (Table 6.2) for equimolar concentrations of SMT, NA-SMT, and NP-SMT fortified together into 1 L water (approximately 1 ng/L each). As a means of determining sulfonamide recoveries, the sulfonamide MALDI-TOF MS responses were measured relative to the internal standard, SMR, which was added to the water sample after the IAC step. The response ratios of each sulfonamide relative to SMR were determined by analyzing the four sulfonamide standards directly by MALDI-TOF MS, in the same proportions as were combined in the water recovery test. The recovery rates for sulfonamides determined here were higher than those reported for water in earlier (Table 5.2) and later work (Table 6.3). This may be because the extraction times for these one litre samples with the HLB Plus cartridge were almost doubled to about 70 minutes in

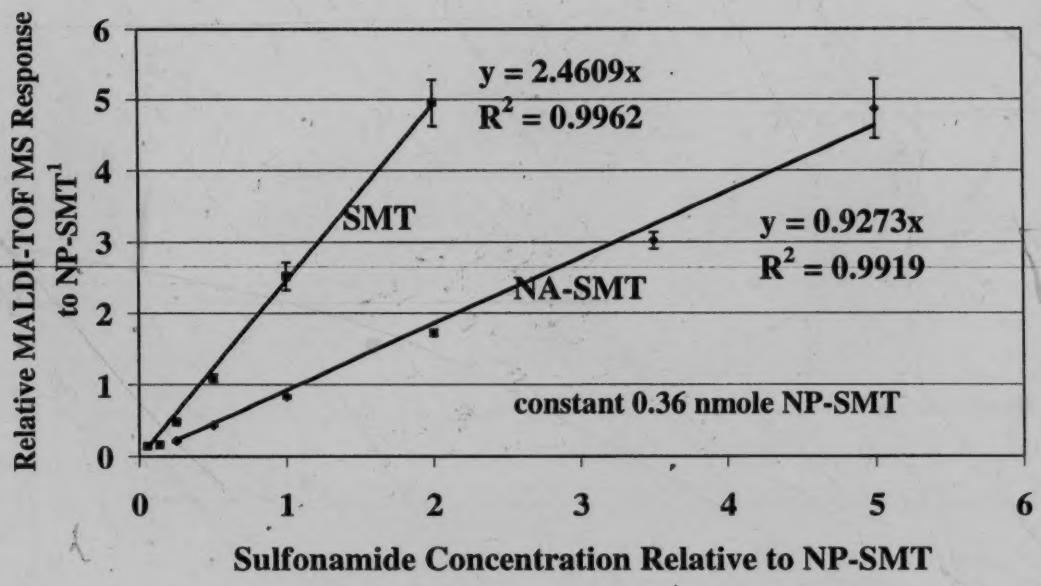

Figure 6.4. SMT and NA-SMT MALDI-TOF MS response relative to NP-SMT

[1] The relative MALDI-TOF MS response is determined for various concentrations of SMT or NA-SMT relative to a constant concentration of NP-SMT (3.6 nmol/10μL representing 0.36 nmol in 1 μL when spotted on the MALDI-TOF MS target) is determined by averaging the values of 3 spectral replicates (the summed response of 100 laser ionization events per spectrum) ± standard error of the mean.

Table 6.2. Percent SPIE[1] recovery[2] of 1 ng/L sulfonamides in water

	SMT	NA-SMT	NP-SMT
Standards[3] directly analyzed	100 ± 7	100 ± 8	100 ± 6
3 step SPIE	84 ± 5	82 ± 1	75 ± 3
2 step IAC & micro HLB	90 ± 12	75 ± 14	75 ± 13

[1]The solid phase immunoextraction (SPIE) system was comprised of three columns: an HLB Plus cartridge, followed by an immunoaffinity column, and then a micro-HLB solid phase extraction column.

[2]Recoveries were determined relative to sulfamerazine (SMR) added to the samples after the IAC step are reported with the standard error of the mean.

[3]SMR, sulfamethazine (SMT), N^4-acetylsulfamethazine (NA-SMT), and N^4-propionylsulfamethazine (NP-SMT) standards were mixed and applied directly to the target to determine the response ratios relative to SMR.

Table 6.3. Solid phase immunoextraction (SPIE) recovery rates estimated for farm samples

Sample	% Recovery [1] for NP-SMT standard	sem [2] (n=3)
Feed	185	14
Weaner pit	90	5
Lagoon 1	170	5
Lagoon 2	70	2
Soil site 1	13	8
Soil site 2	20	0
Pond water 1	65	17
Pond water 2	31	14
Pond water 3	29	3
Well water	37	4

[1] N^4-propionylsulfamethazine (NP-SMT) recovery was determined relative to an sulfamerazine (SMR) internal standard added after the IAC step

[2] sem = standard error of the mean for three spectra used to determine sulfonamide levels for each sample

this experiment. Since the recovery rates of SMT, NA-SMT, and NP-SMT were relatively very close, NP-SMT can be considered a good internal standard candidate for the SPIE system. However, the response ratios of the sulfonamides relative to SMR were determined from only one set of concentrations, and more extensive testing over a range of concentrations is required to establish a linear correlation for future quantification studies. Adjustment factors can be applied for NP-SMT relative to the other sulfonamides if they are found to be consistent.

Water samples were purified by the SPIE system reported in Chapter 4, whereas to compensate for the lower recoveries of soil and manure, a modified SPIE procedure was used involving solvent extraction steps prior to the SPIE procedure. High recoveries of sulfonamides from manure prior to SPE and LC-MS analysis were recently reported, in a procedure which involved a hexane defatting step and ethyl acetate extractions prior to SPE (Pfeifer et al., 2002). These organic extraction procedures were incorporated into the SPIE process for manure and soil samples, and recoveries for NP-SMT were estimated in Table 5.3. The recovery rates varied from being unacceptably low (13% for site 1) to unreasonably high (170% for lagoon 1), and were also inconsistent for a given sample matrix. More development work is needed to achieve both consistency and acceptable recoveries to this system, before it can be considered dependable enough for reliably quantifying sulfonamides in complex sample matrices.

6.4.2. Estimation of Incurred SMT and NA-SMT

The SPIE MALDI-TOF MS system was able to detect incurred sulfonamide in barn manure, both waste lagoons, in soils at both sites, and in low-lying standing water at site 1. A typical positive MALDI-TOF MS spectrum is shown in Figure 6.5, where an analyzed manure sample was positive for SMT and NA-SMT. Peaks were also visible for NP-SMT, the quantitative internal standard, and SMR, the positive control and a reference for recovery rate determination. A quantification estimate was made on the assumption that SMT and NA-SMT were recovered identically to the internal standard, however, more recovery analysis of all analytes and standards fortified into sample matrices is needed to have confidence in this assumption. Regardless, SMT and NA-SMT concentrations were estimated in farm samples using SPIE MALDI TOF MS (Figure 6.6)

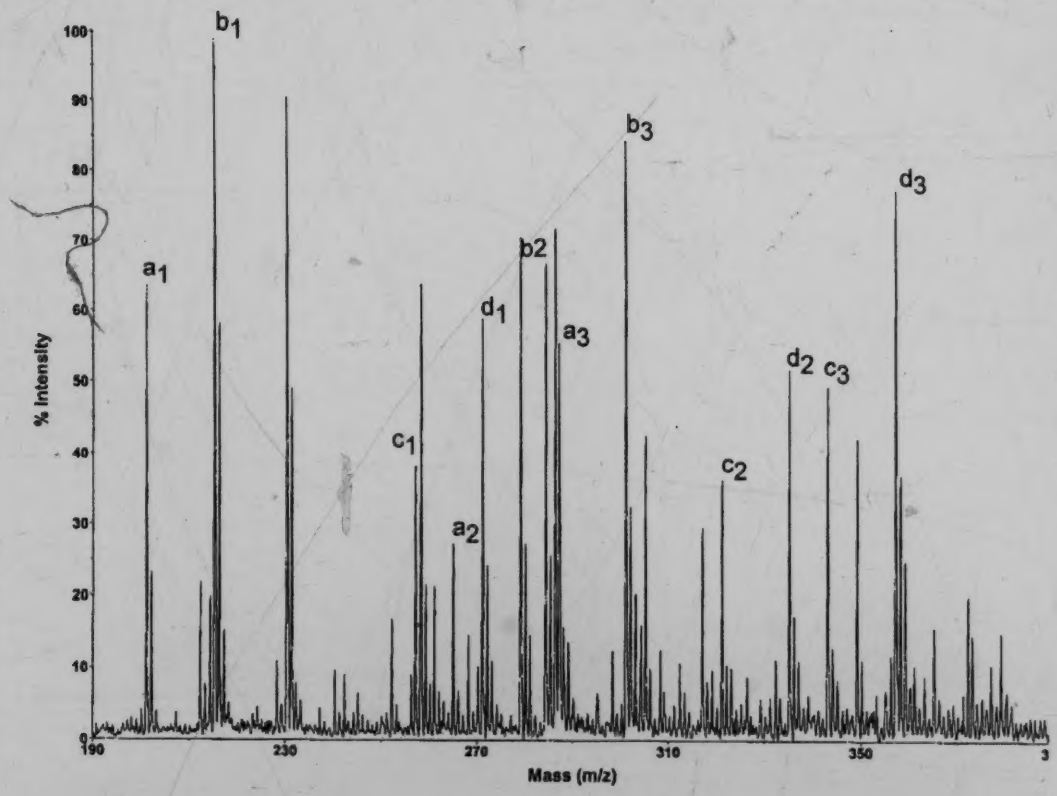

Figure 6.5. A typical MALDI-TOF mass spectrum showing the detection of SMT and NA-SMT from a farm manure sample. Sulfamerazine (SMR), sulfamethazine (SMT), N^4-acetylsulfamethazine (NA-SMT), and N^4-propionylsulfamethazine (NP-SMT) peaks are represented by the letters a, b, c, and d, respectively. The respective sulfonamide fragment and proton and sodium adducts are denoted by the numbers 1, 2, and 3, which follow the letters. SMR and NP-SMT are internal standards. Unlabelled peaks are mainly associated with 2,5-dihydroxybenzoic acid matrix. Three spectra were averaged from each replicate sample to determine sulfonamide levels, and at least two SPIE MALDI-TOF MS determinations were made for each sample. Ordinate scale is an arbitrary % intensity.

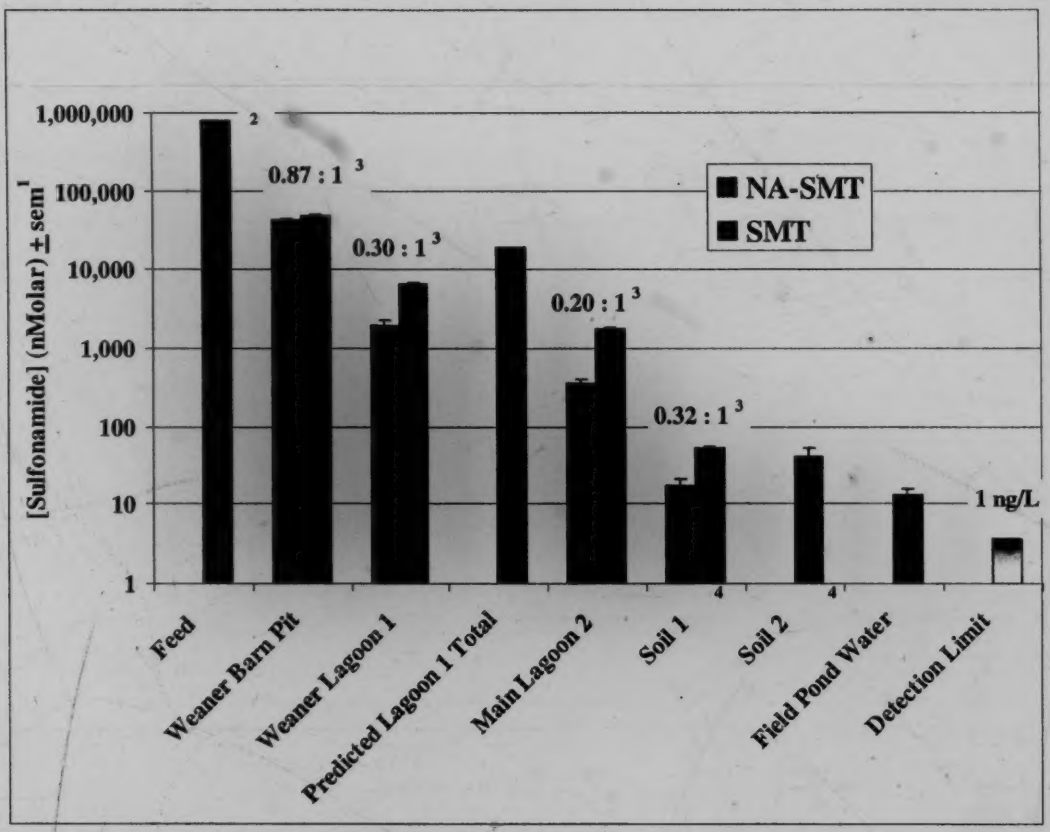

Figure 6.6. Concentrations of SMT and NA-SMT in pig farm samples as determined by SPIE MALDI-TOF MS.

[1] Single samples analyzed in triplicate. Error bars represent the standard error of the mean.

[2] Sulfamethazine (SMT) level reported by the feed supplier.

[3] Molar ratio of NA-SMT:SMT.

[4] Below the detection limit of SPIE MALDI-TOF MS system for these samples (approximately 1 ng/L SMT).

based on their response ratios in standard solutions to NP-SMT. Negative control water, manure and University river bank soil samples as described for the development work of this system in Chapter 5, were tested again with the new SPIE MALDI-TOF MS procedure, and found to be clear of sulfonamides. The concentrations of total sulfonamide followed a logical progression from the highest value in the feed (102 μg/L), and then to sequentially lower levels in the weaner barn pit (26 μg/L), lagoon 1 (2.35 μg/L), lagoon 2 (0.59 μg/L), soils (19.6 ng/L and 11.4 ng/L) and then pond water (3.7 ng/L), as measured in SMT equivalents. The total sulfonamide (SMT + NA-SMT) concentration determined for lagoon 1 was about one half of the estimated value determined by Equation 6.1 (5.5 μg/L). The feed sample taken from the pig barn also had about half the level of SMT reported by the feed supplier (220 μg/L), thus may explain the lower than expected sulfonamide results. It is also possible that sulfonamide in lagoon 1 had bound covalently or non-covalently to sediment, or could have been partially biodegraded. The error is very large in both making the sulfonamide estimate and in determining the sulfonamide concentration in lagoon 1, and the difference in concentration between observed and expected may not be significant.

All positive soil and water samples were in the low ng/L range. One of the two soil samples from each site, one of the two pond samples tested, and the farm well water tested negative. All manure samples resulted in positive MS responses above their limits of quantification[1], whereas positive soil and water samples were only within the system's limits of detection[2] for these particular MALDI-TOF MS spectra. NA-SMT is the main form of the drug excreted from the pig, and is almost exclusively eliminated in the urine at about three times the concentration of SMT (Nouws et al., 1988). The NA-SMT : SMT molar ratios for the barn manure pit, waste lagoon 1, and waste lagoon 2 were 0.87:1, 0.30:1, and 0.20:1, respectively. Interestingly, these NA-SMT : SMT ratios for all environmental samples were less than the 3:1 expected value. Also notable was the

[1] Limit of quantification is defined as the concentration of analyte resulting in a signal 10 times greater than the highest signal of the local background noise, or alternatively, 10 times the standard error of the mean for replicate analyses.

[2] Limit of detection is defined as the concentration of analyte resulting in a signal 3 times greater than the highest signal of the local background noise, or alternatively, 3 times the standard error of the mean for replicate analyses.

progressively decreasing ratio the further "downstream" the waste holding system a sample was taken. It is possible that NA-SMT was deacetylated back to the parent form of the drug. Deacetylation has been extensively studied in humans (Rehm et al., 1986), however, this capability has not been demonstrated by microbial enzyme systems.

6.4.3. Implications of Sulfonamide Findings in Environmental Samples

From an analytical point of view, one important parameter in developing a tool to detect a pollutant in food, water or the environment is to determine how low the detection and quantification limits need to be. Allowable limits for sulfamethazine residues in food products are 100 ng/L for meat and eggs and 10 ng/L for milk. Food analytical systems should be able to quantify a sulfonamide residue at concentrations lower than its allowable safety limits. Limits of quantification are higher than limits of detection, because extra assurances are required above the variation and "noise" associated with the analyte's signal. Food analysis therefore requires a detection limit of about 1 ng/L or less.

The relevance of sulfonamides in the environment is not clear, and therefore, the safety limits or the detection and quantification limits required from analytical tools are not known either. Although there are currently no environmental regulations controlling sulfonamides or pharmaceutical pollutants, growing concern over the synergistic effects of an increasing number and quantity of persistent pollutants in the environment are compelling governments and researchers to establish environmental safety guidelines. In Chapter 5, it was reported that the SPIE MALDI-TOF MS system was able to detect 0.1 ng/L SMT or NA-SMT in fortified water samples and at low ng/L-levels in and soil and manure samples. These detection limits for SPIE MALDI-TOF are more than adequate for monitoring sulfonamides at levels found directly in manure samples. However, if SPIE MALDI-TOF MS is to track the fate of diluted sulfonamides in the environment, improvements in assay reliability are required in the low- to sub-ng/L range for various complex environmental sample types.

The main area of concern for sulfonamides released to the environment is the promotion of conditions for the natural selection of sulfonamide-resistant strains of microorganisms, and perhaps more relevantly, the concomitant selection for multi-drug resistance when these traits are linked on bacterial plasmid DNA (George, 1996; Sunde

and Sorum, 2001). The sulfonamides fed to the pigs in this pilot study had apparently been diluted more than 1000 fold by the time they reached the soil or field pond. These concentrations are relatively low, and are lower than the allowable limits for SMT residues in food. Also, the low ng/L levels found where the manure was spread were below the limit required for sulfonamides to cause a bacteriostatic effect in laboratory situations (Mengelers et al., 1989b; Mengelers et al., 1997), and therefore, would theoretically not select for sulfonamide resistant bacteria at these levels. However, the SMT concentrations in the manure pit and lagoon 1 were of concentrations that may have been capable of exerting some selection pressure on microbial populations in the fermenting waste (low µg range) toward sulfonamide-resistance over extensive time. Alternatively, based on indirect findings from recent *in-vitro* research on sulfonamide degradation in activated sludge (Ingerslev and Halling-Sorensen, 2000), it is possible that lagoon bacteria could be conditioned over time to degrade sulfonamides.

A further concern in the farm results was the evidence for deacetylation of NA-SMT to the microbially active form in the waste lagoons. There, NA-SMT occurred as much as five fold lower than SMT, even though it was expected to be three times as high if represented in the proportions excreted. Previous evidence for the possibility of environmental deacetylation of sulfonamide metabolites was provided by research that monitored the hydrolysis of N-acetylsulfadiazine added to manure (Berger and Brünung-Pfaue, 1986), or by the observation of N^4-acetyl residue concentrations lower than the parent sulfonamide in manure (Haller et al., 2002). NA-SMT does not have bacteriostatic activity, and deacetylation causes the antimicrobial form of the drug to be regenerated, which may have important environmental implications. If deacetylation of the main excreted form, NA-sulfonamide, occurs to a significant degree, then microbiologically active sulfonamide levels in waste lagoons could be maintained at high levels. Also, depending on the climate, the land topography, soil types, and farming practices, farm lagoon waste spread onto soils to fertilize crops may result in dramatically different sulfonamide concentrations in soil and water microenvironments of agricultural fields, and may even sometimes be concentrated in micro-environments affecting individual plants or groups of plants.

From an analytical perspective, any method for residue analysis needs to monitor a large range of concentrations, spanning a factor of over a million, from sub-ng/L to high µg levels. Currently, the SPIE MALDI-TOF MS system depends upon proper dilution and targeting of the sample volume analyzed, so as not to overload the IAC capacity. Although initial research has demonstrated that the recoveries of closely related sulfonamides are similar under saturating conditions, quantification and recovery estimate errors would be introduced if the IAC were operated significantly beyond its capacity. This problem can be addressed by splitting samples and spiking them with high or low concentrations of internal standard, allowing the IAC to be overloaded in the case of high level samples, and to validate consistent recovery ratios of the SPIE based on saturated and non-saturated conditions. Another consideration is adequately diluting a sample so that the final analyte concentration can be analyzed within the dynamic linear response range of MALDI-TOF MS, usually not more than a range of two logs of ten for the on-probe analyte concentration. Other MALDI ionization MS systems are available with a larger dynamic response range, and will be discussed in the conclusion chapter.

6.5. SUMMARY

The SPIE MALDI-TOF MS system demonstrated a capability to simultaneously detect an incurred drug and its main metabolite (SMT and NA-SMT) at the low ng/L level in environmental sample matrices. Confidence in quantification by this methodology was not established due to low and variable recoveries from environmental sample matrices. However, analyte identities were confirmed by acquiring at least 3 mass peaks associated for each sulfonamide in response intensity patterns diagnostic for sulfonamides. Although significantly more developmental research is necessary to validate this method, concentration estimates were made for farm samples relative to a new internal standard used for the first time in sulfonamide analysis (NP-SMT). These measurements were reasonable in relation to predictions based on total sulfonamide and volume inputs to the weaner waste lagoon. A progressively decreasing SMT : NA-SMT ratio was observed in samples taken further "downstream" the waste holding system, implying that the inactive acetylated metabolite of SMT (NA-SMT) may be deacetylated,

selectively bound to solids in the waste material, or converted to some unrecoverable form. The ability of SPIE MALDI-TOF MS to detect both SMT and NA-SMT incurred by normal agricultural practices suggests it is a suitable means of analyzing trace level sulfonamides. To improve the reliability of this system, improvements are required in the initial liquid/liquid or SPE extraction and recovery of sulfonamides. Coupled with immunoaffinity chromatography, higher recoveries would allow SPIE MALDI-TOF MS to achieve the consistency and sensitivity necessary to be validated for drug quantification at the trace level.

Chapter 7

CONCLUSION

7.1. INTRODUCTION

The conclusion starts with a specific summary of thesis research accomplishments with respect to the objectives (section 7.0), and then the significance and contributions of this work to science are discussed (section 7.1). Next, a synopsis presents the improvements required of solid phase immunoextraction (SPIE) coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to actualize its full potential in residue analysis of sulfonamides and other small molecules in complex sample matrices (section 7.2). This research is then placed in perspective relative to trends in analytical chemistry and what it means for the future of residue analysis from a technological perspective (section 7.3). Lastly, the implications of future advancements in residue analysis – as they impact scientific and moral assessment of risks and benefits - will be considered, specifically, with respect to sulfonamides, and more generally, with respect to all organic compounds used by medicine, agriculture, and industry (section 7.4).

7.2. THESIS SUMMARY

Thesis objectives were addressed in chapters two through five and are summarized in two parts below.

7.2.1. Development, Characterization, and Evaluation of Sulfonamide-Specific Antibodies for Immunoaffinity Chromatography

The specific goals of Chapters 3 and 4 were to develop and purify sulfamethazine-(SMT) and sulfathiazole- (STZ) specific polyclonal antibodies, to make immunoaffinity columns (IACs) from purified antibodies, and to evaluate the ability of ELISA analysis of sera to predict their subsequent immunoaffinity column (IAC) performances.

Ten different rabbit anti-sulfonamide sera were developed, the sulfonamide-specific antibodies were purified, and IACs were made with some of these antibodies. As expected, linker arm effects were observed for the antibodies in both ELISA and IAC. Linker arm effects were measured as a function of the cross-reactivity of the antibody to sulfonamide derivatives or conjugates more structurally similar to the immunogen than

the parent sulfonamide hapten. Linker arm effects were greater for the smaller hapten sulfathiazole. When comparing the sulfonamide immunogens made with succinyl- or azo-linker arms, the succinyl system had greater overall linker arm effects. Linker arm effects were more specific for antibodies from the azo- compared to the succinyl-linker arm system, and the titres of sera from the azo linker arm system were substantially greater than the succinyl linker arm system.

Experiments were able to describe the most important and average structural features of the sulfonamide haptens on the immunogen, including the linking arm, that conferred sulfonamide-affinity by polyclonal antibodies. The average size of the sulfonamide hapten - determined from ELISA cross-reactivity studies with sulfonamide derivatives – was estimated to involve the complete parent molecule (SMT or STZ) and a portion of the linker arm (diazo part or the carbonyl part of the succinyl linkage), but likely did not involve further aspects of a sulfonamide's attachment to the immunogen protein, *Limulus polyphemus* hemolymph. Based on this estimate for hapten size, the average anti-sulfonamide binding pocket was predicted to be narrow and relatively deep, with dimensions of approximately 6-7 Å deep and 3-4 Å across. Also, it was predicted that high affinity anti-sulfonamide antibodies would possess a positively charged lysine or arginine amino acid residue in one of the hypervariable sequences at the binding site to stabilize the negatively charged sulfonamide.

This research demonstrated that competitive ELISA was more sensitive and responsive than IAC to immunogen linker arm effects and structural differences between sulfonamides. This has two unexpected and practical consequences. First, regardless of the specificity conferred to sera generated by using immunogens designed for sulfamethazine specifically, IACs were able to bind a wide range of sulfonamide compounds and sulfamethazine derivatives under non-saturating conditions. Sulfanilamide, the smallest sulfonamide available, was the only one tested that was not recovered by IAC. This generic principle seen for anti-sulfonamide antibodies in IAC is significant because it predicts that, under non-saturating analyte conditions, antibodies designed to be drug-class specific (for instance, specific to the end of the molecule in common with all sulfonamides) may be even more accommodating to a broader range of sulfonamides when used for IAC analyte extraction, in non-saturating and possibly

saturating conditions. This opens practical opportunities for generic-drug IACs; previous ELISA work by others on class-specific antibodies against haptens could be reevaluated from an IAC perspective. Secondly, the greater responsiveness of ELISA to linker arm effects was determined to be a potential serum screening tool, prior to sacrificing animals during the immunization regime. It allowed evaluation of polyclonal antibodies by predicting their subsequent anti-hapten IAC performance. For analyte conditions that saturate IACs using sulfamethazine-specific antibodies, ELISA analysis of sera was able to predict both the non-competitive IAC capacity for related sulfonamides, and the competitive IAC cross-reactivity for sulfamethazine derivatives. This is an important insight, both in allowing the proper selection of polyclonal antisera before investment of time and resources in anti-hapten IAC development, but also as a screening criterion in the selection of hybridoma cell lines in monoclonal antibody production, or in recombinant antibody fragment candidate selection in phage or ribosome display technologies.

In developing an anti-SMT IAC using CNBr-activated Sepharose 4B, problems of antibody "bleed", analyte breakout and column collapse had to be addressed (see appendix, Chapter 9). After a more robust hydrazine solid phase support alternative was unsuccessfully tested, a CNBr-activated Sepharose 4B gel was used for further IAC development. High IAC loading capacities were achieved by using an immunopurified, sulfonamide-specific fraction from sera. Excellent IAC high loading capacity was achieved (100-220 nmol or 28-61 μg SMT/mL IAC gel), which was associated with high on-column antibody purity and activity (60 - 100% theoretical binding capacity assuming all protein on IAC was IgG antibody). The IACs were robust and reusable; in some cases they were used and regenerated with acid over 100 times without noticeable loss of performance. To overcome the problems associated with high volume samples, a combination of IAC and solid phase extraction (SPE) proved to be the best option in concentrating and purifying sulfonamides.

7.2.2. Development of Solid Phase Immunoextraction (SPIE) with MALDI-TOF MS for Detection of Fortified and Incurred Sulfamethazine Residues in Environmental Samples

The goals of Chapters 5 and 6 were to develop a technique combining immunoaffinity chromatography with MALDI-TOF MS to detect fortified and incurred residues in farm-type environmental matrices at the low ng/L-level. An anti-SMT IAC ("1B" from SMT-azo-LPH immunogen) was determined to have adequate affinity, capacity and recovery performance for purifying sub-ng/L concentrations of SMT and NA-SMT environmental samples when coordinated with primary and final non-specific solid phase extraction columns, and together were referred to as solid phase immunoextraction (SPIE).

SPIE was comprised of three separate stages; first, solid phase concentration with HLB Plus™ SPE cartridge, then immunoaffinity chromatography with an anti-SMT IAC, and lastly, a concentration using a micro-HLB column before MALDI-TOF MS analysis. As an adaptable model for other drugs and haptens, this research described the recovery and detection of ng/L-levels of sulfamethazine and its N^4-acetyl metabolite fortified into water, or in soil or manure suspensions, using solid phase immuno-extraction (SPIE) coupled with MALDI-TOF MS (Chapter 5). The limit of detection for SMT and NA-SMT was 0.1 ng/L in fortified water and in the low ng/L range in various fortified soil and manure matrices. The standard, sulfamerazine (SMR), acted as an internal positive control and enabled the determination of recovery rates. The SPIE process took about 1.5 h per batch (8-24 samples/batch) for sample enrichment, 5 min per batch for probe preparation, and 5 min per sample to acquire and process the spectrum, representing two or three-fold improved analysis efficiency compared to other conventional and definitive methodologies.

The next phase in testing the assay was in analyzing sulfonamides incurred in farm site environmental samples (Chapter 6). Manure, soil and water samples were obtained from a local pig farm with a 23-year history of using sulfonamides and spreading the manure on nearby fields. SPIE MALDI-TOF MS was able to detect both SMT and NA-SMT in all waste lagoon samples, and in some of the soil and water samples. Although significantly more development research is necessary to validate this

as a quantitative tool, farm sample quantification estimates were made relative to a new internal standard, N^4-proprionylsulfamethazine (NP-SMT). Also, sulfamerazine (SMR) was added after the IAC step of the SPIE process, acting as a positive control by adjusting for the large variability in MALDI-TOF MS response, and also as a reference to determine sulfonamide SPIE recovery rates. Confidence in quantitative estimates made by this methodology was not established due to low and inconsistent recoveries. The total sulfonamide concentration determined for incurred samples was approximately one half relative to an estimate using total sulfonamide and volume inputs to the weaner waste lagoon (2.4 µg/L versus 5.5 µg/L). This recovery was a reasonable outcome, especially since sulfonamides have been shown to bind to proteins, and likely could have adsorbed significantly to settled sludge in the farm lagoon. The chemical stability of sulfonamides makes biodegradation an unlikely alternative explanation.

Progressively decreasing SMT: NA-SMT ratios were observed in samples taken further "downstream" the waste holding system, implying that the inactivated acetylated metabolite of SMT, may be either deacetylated or selectively bound to solids in the waste material. The low ng/L-level sulfonamides found in one of each of the two water and soil samples indicate that the total dilution observed from the first lagoon to the field was approximately 100 – 500 fold. This dilution estimate is not considered one that could be used elsewhere to predict sulfonamide dilution into the environment by farming practices, because it represented only one set of samples in time under specific soil and weather conditions, and was not derived in a randomized manner necessary for a valid survey. However, it does represent the first incurred finding of sulfonamide in soil by normal agriculture practices.

SPIE MALDI-TOF MS system demonstrated its capability to simultaneously monitor a drug and its main metabolite, and to confirm sulfonamide identity by acquiring a reproducible response pattern involving 3 adduct and 1 fragment peaks for each sulfonamide, coordinated with the efficient sulfonamide-selective filter of the IAC system.

7.3. CONTRIBUTIONS TO THE ADVANCEMENT OF SCIENCE IN THE FIELD OF RESIDUE ANALYSIS

This work represented an important contribution to science in that it was the first demonstration of MALDI-TOF MS - a relatively new and highly efficient analytical technology - to measure trace-level, small molecule (<500 Da) residues in complex sample matrices (Chapter 5). The high throughput potential of MALDI-TOF MS, combined with its tolerance of sample impurities, made it an excellent candidate system to combine with another technology with similar efficiency potential. Together with solid phase extraction, immunoaffinity chromatography was capable of adequately enriching ng/L-levels of sulfonamides from complex sample matrices for subsequent MALDI-TOF MS detection. This system is proposed to be capable of overcoming the conventional bottleneck limitations associated with on-line liquid or gas chromatographic sample purification prior to MS analysis. This could transform confirmatory residue analysis output capabilities from hundreds to thousands of samples per day.

Although this research focused on developing greater efficiency in analyzing sulfamethazine residues in farm-site environmental sulfonamides, its application was much broader in scope. This model, although having many considerations specific to the chemistry, activity, metabolism, and detection of sulfamethazine, could be applied to other small organic molecules. For example, other drugs such as tetracyclines, steroidal hormones such as estradiol or androgens, or to pollutants such as dioxins, could be analyzed by a similar procedure. Also, although water, soil, and manure environmental samples were examined, the analysis of these sample matrix models could theoretically be adapted to other matrices such as liquid and solid foods or to industrial product and waste processing systems.

This work also represented other "firsts" in science. It represented the first time to detect incurred sulfonamides in farm soil samples as connected to normal agricultural practices. It was the first to show both an approximate accounting for the total sulfonamide in large farm waste lagoons, and the sequential downstream fractional reduction to the main metabolite relative to the parent sulfonamide in environmental samples. Both of these findings have strong implications for the possible environmental

persistence of sulfonamides, and of the possible reactivation of sulfonamide metabolites to the bacteriostatic parent form.

Although monoclonal antibody technology is the most common commercial antibody source for use in hapten IAC, polyclonal antibodies still have distinct advantages of high affinity, broad diversity, and a low cost to initially produce, and these attributes are responsible for their continued and common use for this technique (Stevenson, 2000). Despite the long-term use of polyclonal antibodies in hapten IAC, this research represents one of the first reported uses of a hapten-specific polyclonal antibody fraction for high capacity immunoaffinity purification (Ben Rejeb et al, 1998 was the other, but they used a different hapten for immunization than for immunopurification of polyclonal antibody, to overcome the problems associated with extremely high affinity to the immunogen hapten-linker arm system). More research will be required to establish if the success of this system was due to the unique acid-base chemistry of sulfonamides, and if it is applicable to other haptens. Also, important considerations of polyclonal antibody performance (ELISA evaluation of linker arm effects, specificity, cross-reactivity, relative affinity, etc) were considered extensively and thoroughly for the first time for a hapten model system in relation to subsequent immunoaffinity column (IAC) performance. These findings have relevance not only to polyclonal antibodies selection for use in IAC, but also to newer technologies such as monoclonal antibody and recombinant antibody development, also capable of being used in IAC.

7.4. FUTURE IMPROVEMENTS AND POTENTIAL OF SPIE MALDI-TOF MS

Improvements in the analysis system can be addressed at both the SPIE and the MALDI-TOF MS parts of the procedure. In the SPIE procedure, especially for the solid sample matrices, procedural and technical changes are required to afford consistent and higher sulfonamide recoveries. Once the recovery issue is addressed, then work can proceed in optimizing overall assay sensitivity and precision, and blinded accuracy assessments can be made in the quantitative development of the system. Following the development of a valid quantification system, improvements can be made in analysis

speed, taking advantage of the inherent efficiency potential of coupling IAC with a MALDI-ionization source prior to MS detection.

7.4.1. SPIE Recovery Improvements

The greatest limitation of the current system is poor recovery of analytes, especially from solid matrices like soil and manure. The results indicate improvements in recovery rates and precision will be achieved by focusing on the initial extraction and concentration of sulfonamides before IAC and micro-SPE. Some ways to improve initial sulfonamide recovery are as follows:

1. Decrease extraction rates during SPE of water or aqueous extracts. Halving SPE loading rates from 25 mL/min to 12 mL/min was shown to improve sulfonamide recoveries substantially in one experiment. However, this also significantly decreases the efficiency of the process.

2. Use a SPE system with higher affinity and less breakout of sulfonamides, perhaps based on ion exchange principles of extraction. Similar to the hydrophobic-lipophilic balanced system of HLB Plus™ sorbent used in SPIE, a system like the Oasis MAX™ sorbent (Waters, Milford, MA), may adsorb sulfonamides based on both hydrophobic and coulombic interaction, as well as hydrogen bonding at pH >7 (see Figure 6.1). This strategy is likely to overcome the problems associated with poor sulfonamide recovery from aqueous solutions.

3. Investigate options of matrix solid phase dispersion using the hydrophobic/ionic interaction sorbent recommended above. Low moisture or freeze-dried manure or soils samples could be mixed with sorbent material using appropriate minimal buffer to extract and elute sulfonamides. This technique has been applied successfully to sulfonamides in food sample clean-up (Kishida and Furusawa, 2001).

4. Investigate, refine, and optimize the details of solvent extraction for solid samples such as manure and soil, prior to SPE. Solvent extraction or the solvent and buffer systems used in liquid/liquid extraction, have shown potential for good recoveries in other research when properly applied and coordinated with SPE (Pfeifer et al., 2002). This aspect of extraction can be miniaturized and easily scaled up using commercially available batch processing technologies.

7.4.2. MALDI-TOF MS Sensitivity Improvements

Sensitivity can also be improved by addressing the sample preparation as it affects the sulfonamide ionization in MALDI-TOF MS detection. Too much internal standard can interfere with ionization of analytes at relatively lower concentrations, and can saturate the MALDI-TOF MS detector in the low mass range. Further work is required to optimize the MALDI matrix and internal standard concentrations relative to the incurred sample sulfonamide. Also, co-crystallization of sample and matrix to provide a more consistent and homogeneous crystal bed should be investigated with respect to optimizing signal to noise ratio and resolution. New advancements in instrumentation are decreasing the lower sensitivity limits. When a MALDI ion source is coupled in tandem with quadrupole MS filter systems prior to TOF to minimize the large chemical background associated with matrix ions in the low mass range, on-probe detection limits as low as 6 ng/L have been achieved (Hatsis et al., 2003). This is about 40 times more sensitive than MALDI-TOF MS alone (Ling et al., 1998).

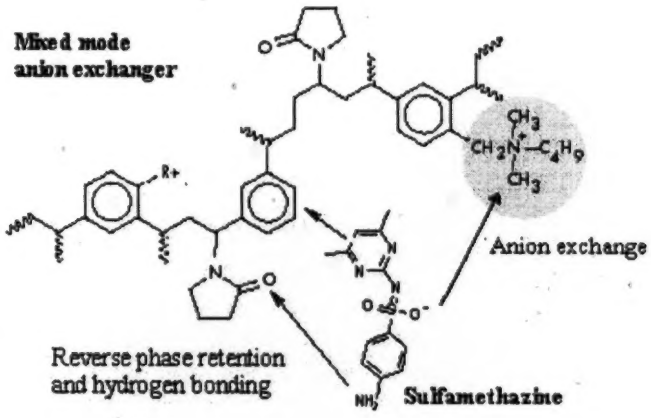

Figure 7.1. A mixed mode anion exchanger interaction with sulfamethazine (Adapted from Waters Corporation technical bulletin; www.waters.com accessed March, 2004).

7.4.3. Considerations to Improve Quantitative MALDI-TOF MS

To make SPIE MALDI-TOF MS a quantitative method, the following issues need to be addressed:

1. ***Internal Standard:*** The ^{13}C isotope of sulfamethazine (MW = 284.1), containing six ^{13}C atoms in the benzene ring, is the ideal internal standard, and is commonly described by official methods (Official Methods of Analysis of AOAC International, 2000). This standard isotope could be used in SPIE-MALDI-TOF MS if a matrix other than dihydroxybenzoic acid (DHB) were used due to the conflicting DHB peak at *m/z* of 284. However, other matrices may not have adequate signal-to-noise ratio, as was seen in this preliminary research and by others (Ling et al., 1998). N^4-propionylsulfamethazine (NP-SMT) was an adequate standard, and behaved similarly as the analytes in IAC. Any internal standard selected will need to be equivalent or reproducible in SPIE recovery rate and MALDI-TOF MS response relative to other SMT metabolites like N^4-acetylsulfamethazine. Other metabolites like hydroxyl, sulfate, and glucose conjugates and desaminosulfonamides, although rare, still need to be addressed as well with respect to the analysis system's ability to include them in detection and quantification.

2. ***Dynamic Linear Response Range:*** Critical control points need to be identified to maintain MALDI-TOF MS linearity of response for matrices at various concentrations, under various instrument settings, and at various internal standard concentrations. The linear response range of the Bruker Proflex III MALDI-TOF MS instrument was limited to a detection range within one or two orders of magnitude, so proper sample dilution was important in targeting an acceptable analyte quantitative concentration zone. The ability of MALDI-TOF MS to respond in a linear fashion for a range of analyte concentrations is a function of the ionization conditions, but is also a function of the instrument. For instance, experiments quantifying pharmaceutical drugs using a MALDI-triple quadrupole instrument, resulted in a dynamic range of calibration covering at least three orders of magnitude (Hatsis et al., 2003).

3. ***Variability and Precision:*** It is important to optimize the instrumentation and parameters to minimize the inherent variability associated with the MALDI ion source. For instance, high laser pulse frequency has been demonstrated to increase the number of

accumulated spectra per sample and to reduce the standard deviation (SD) in analyte relative response to 5% from a more commonly reported operating SD of 10-20% (Hatsis et al., 2003).

4. ***Instrument Settings for Spectral Acquisition:*** For any given instrument it is important to standardize the operational parameters used in acquiring and accepting/rejecting spectra. Laser settings, delayed extraction and accelerator timing and voltages, focusing lens settings, detector voltages, and numbers of laser shots per sample can all be set for an analysis, but usually have to be adjusted from sample to sample, and even within a sample over time, in relation to how the particular sample is responding. Sample matrix effects and inconsistent, non-homogeneous crystallization of an analyte with the MALDI-TOF MS matrix cause much of the response variation seen in this technique. An experienced operator scans a spot and adjusts the instrument settings according to how the sample is reacting. Automated programs are becoming more sophisticated, and are necessary to replace the manual operator for high throughput systems (Brown et al., 2002; Nicola et al., 1998b).

7.4.4. Considerations for Improvements in Specificity of SPIE Clean-up

Immunoaffinity chromatography acts as a primary screening prior to MS analysis. The confirmatory ability of coupled methods, using different principles of separation, is greater than either system separately. The SPIE system served to extract SMT, NA-SMT, and NP-SMT; their associated masses were then detected by MALDI-TOF MS and interpreted with reasonable assurance. The specificity of the SPIE system therefore determines the exclusiveness of this step. However, IAC specificity is also a limitation because it cannot monitor structurally dissimilar analytes that are not bound by the IAC antibodies. An advantage of other systems that use non-exclusive initial clean-up steps like SPE, followed by LC-MS, is that they are capable of multi-residue analysis (Doerge et al., 1993). IAC could still be used for multi-residue analysis if a sample were passed serially through several drug class-specific IACs. Each drug class could then be separately eluted and analyzed in parallel. This IAC battery format has been investigated successfully for immunochromatography of various cytokine compounds (Phillips and Krum, 1998). Otherwise, mixtures of drug class-specific antibodies could be attached to

one column, however, this would greatly limit the IAC loading capacity to any one drug class.

Polyclonal antibodies do not perform consistently, and each batch has to be completely recharacterized, repurified, and restandardized (as demonstrated for batch 1 and 2 immunizations, which ostensibly received both the same immunogens and the same immunization regime). This disadvantage of using polyclonal antibodies can be overcome by developing monoclonal or recombinant antibodies, which can be produced in virtually unlimited quantities. Although they have a greater initial cost, once a monoclonal antibody is obtained with the proper binding characteristics, it can be consistently and reproducibly used in IAC, with less expense to produce and control in the long term. If well-defined and highly specific monoclonal antibodies or recombinant antibody fragments were to be used in IAC, the confirmatory nature of subsequent MALDI-TOF MS would be greater, albeit with limitations in multi-residue analysis. Although monoclonal or recombinant antibodies inherently are more specific, they can be selected for the ability to cross-react to a class of compounds by designing the screening methods using haptens that share structural similarities with the whole class of compounds. Regardless, polyclonal antibodies are inherently better candidates for generic-type specificity or broader cross-reactivity, because their heterogeneous nature implies they exist as the sum of a variety of high affinity binding strategies, rather than a singular compromise, as with monoclonal antibodies.

The specificity of the polyclonal anti-SMT IACs in this research was found to be dependent on the saturation of the IAC column. These polyclonal antibodies were designed for SMT-specificity, yet were equally able to recover a wide variety of other sulfonamide types as long as the column was not saturated. As soon as the IAC was saturated with haptens though, specificity biases were manifested sequentially based on similarity to the immunogen. Although resulting in limited success for ELISA, it is proposed that attempts to make generic polyclonal and monoclonal antibodies to sulfonamides by other research teams (Haasnoot et al., 2000a; Sheth and Sporns, 1991; Spinks et al., 2002), would be ideally applicable in generic sulfonamide IAC. Since IAC was shown to have less dramatic linker arm and sulfonamide-structure specificity than

corresponding use in ELISA, future research is indicated for sulfonamide-specific, and more broadly, generic-drug IAC systems.

7.4.5. Considerations for Improving the Confirmation of Analyte Identity in MALDI-source MS systems

Although MALDI-TOF MS was able to acquire a reproducible response pattern involving 3 adduct and 1 fragment peaks for each sulfonamide, the standard for MS instruments is usually to scan for 3 separate fragments. Since MALDI-TOF MS uses a "soft" ionization process, its advantage for macromolecular analysis is that it does not cause fragmentation. To improve confirmatory nature of MALDI-TOF MS fragmentation can be emphasized by increasing the energy of the laser response, but in the case of sulfonamides, only one fragment associated with the analyte was seen. Alternatively, the reflectron mode of operation can be used to monitor for further analyte fragmentation due to post source decay. The "gold standard" in confirmatory MS is still found though, in the coupling of tandem mass spectrometers. For MALDI source systems, this serves to filter out interfering matrix ions and to induce and measure analyte fragmentation pattern. MALDI-TOF MS is currently commercially available coupled to a quadrupole mass filter and a collision cell to generate fragments for high resolution MS/MS confirmation of analyte identity (Micromass Q-TOF Ultima™ MALDI, Waters Corporation, Milford, MA). Similar systems have already been applied to high throughput identification of small pharmaceutical compounds (Hatsis et al., 2003).

7.4.6. Considerations for Improvements in Analysis Efficiency of SPIE MALDI-TOF MS

In mass spectrometry, high throughput analysis is currently associated with systems designed for in-line SPE and HPLC or GC. Advances in valve switching technology allow for more than one SPE or HPLC separation to occur, out of phase, simultaneously, coordinated with direct coupling to MS analysis. However, these systems are fundamentally limited by expensive chromatographic separation and are not practical to run in parallel in any significant number. Relative to the potential of MALDI-TOF MS, they cannot be considered high throughput systems because they analyze at

most one or two hundred samples per day. Commercial MALDI-TOF MS systems are designed for high throughput analysis (thousands of samples or spectra per day). Current MALDI source instruments coupled with quadrupole-time-of-flight or triple quadrupole mass spectrometers, have employed high repetition rate lasers capable of 100-fold higher pulse frequency compared to regular MALDI lasers, resulting in quantitatively scanning a sample in 15s, or 24 min per 96 spot probe (Hatsis et al., 2003). Especially when coupled with automatic probe spotting, instrument loading, and data acquisition/processing systems, MALDI-TOF MS is generally not the rate limiting step of analysis.

The key to further improving mass spectrometric residue analysis is to focus on pre-MS aspects of analysis by miniaturizing and automating large scale, parallel, off-line sample handling and clean-up. These potential improvements are particularly suited to coordination with automated probe spotting and application to high throughput MALDI-TOF MS due to its tolerance of sample matrix impurities, thus lending it to be coupled with less rigorous separation technologies.

MALDI-TOF MS systems are capable of spotting sub-nanolitre quantities of sample (Little et al., 1997c; Onnerfjord et al., 1999), representing more than 1000 fold decrease in end volume required than the current protocol that spots 1 µL of a 10 µL end sample volume. This 1000 fold gain could be actualized in any combination of decreased initial sample volume required or increased assay sensitivity (i.e. increased sulfonamide concentration spotted on the probe). This means that if the samples analyzed were 1 mL instead of the current 1 L, many automated options would be available for sample processing.

Commercially available, disposable micro-SPE cartridges are used in automated sample extraction systems and look like 96 well microtiter plates (Figure 7.2). Theoretically, this format could also be used for IAC, and could be coordinated in tandem with similar micro-SPE systems to concentrate and purify drugs from biological samples prior to probe spotting. Miniaturization also allows the use of smaller IAC systems, making them disposable, thus eliminating time and contamination risks associated with IAC regeneration. Another IAC modification to increase efficiency, would be to use solvent elution systems that would make the post-IAC step redundant. In the current SPIE protocol, a post IAC micro-SPE step is used to switch solvent systems from the

aqueous eluant of IAC, to a volatile system like ethanol. The ethanol is then quickly evaporated prior to mixing with minimal volume of MALDI-TOF MS matrix using heat and nitrogen gas. Alternatively, with a disposable IAC system, the water could be vacuum dried completely from the system and the gel bed could even be collapsed. The analyte would then be eluted in minimal volume of organic solvent for drying using flash, rotary evaporation systems, or eluted directly for application to the MALDI-TOF MS

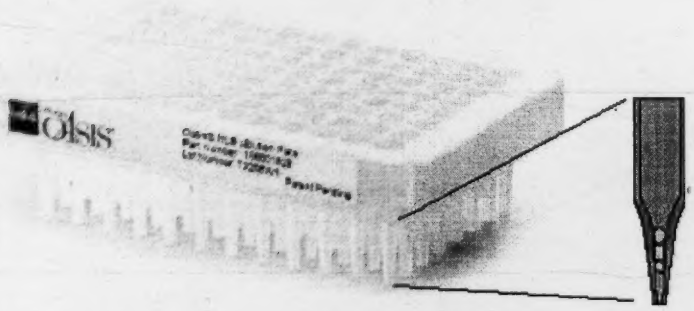

Figure 7.2. Commercial micro-elution technology used for SPE of drugs. 96 well array of sorbent materials can be eluted in µL quantities of solvent. A longitudinal cross section is shown of a single micro-SPE chamber containing the sorbent (Adapted from Waters Corporation technical bulletin; www.waters.com accessed on March, 2004).

probe with a solvent already containing DHB matrix. Miniaturization of sample addition to MALDI-TOF MS probes has demonstrated that handling and spotting small volumes may not be major limitations (Foret and Preisler, 2002a; Little et al., 1997b).

Other immuno-chromatography improvements contributing to analysis efficiency include increasing the IAC analyte loading capacity so that the column size can be diminished, thus minimizing elution volume. In this research, immunopurification of sulfonamide-specific antibodies served to create as much as a 10-fold increase in sulfonamide binding capacity. No sign of steric hindrance was observed at antibody incorporation rates that saturated the sepharose gel, likely because the sulfonamide hapten is relatively small. Further gains in loading capacity could be achieved by using smaller fragments of the antibody, which still contain the binding site. For example, if the Fab antibody fraction were used – obtained by enzymatic digestion of IgG – a

theoretical 3-fold increase in loading capacity is possible over specific IgG. Similarly, if recombinant antibody fragments are used, such as the variable domain of light and heavy chains (Fv) or single chain Fv (ScFv), a theoretical 6-fold increase is possible. It has been shown that some monoclonal antibodies only use one of the variable domains (V_H) in binding a small hapten (Murata et al., 2002), and camels have a naturally occurring single-domain heavy chain (Spinelli et al., 2001). In either case, if V_H antibodies were obtained against a drug, they could be used to effect a 12-fold increase in loading capacity over the analogous IgG molecule. Using recombinant fragments, IAC loading capacity could be improved by more than 100-fold over the common values sited for non-enriched polyclonal antibodies, or more than 10-fold better than monoclonal antibodies or hapten-purified polyclonal antibodies. With these improvements the theoretical IAC loading capacity for compounds like sulfamethazine could reach 600 μg or greater per mL solid phase gel, similar to the corresponding SPE capacity using conventional non-specific solid phase packing sorbent materials. At these loading capacities, the final concentration of analyte eluted from an IAC may be high enough so that SPE concentration is not needed.

7.5. THE FUTURE OF RESIDUE ANALYSIS: A TECHNOLOGICAL PERSPECTIVE

Advancements in analytical chemistry are largely related to the rapid changes taking place in computers, not just in terms of computer control of instrumentation and spectral processing, but more importantly, in how scientific knowledge can be stored, accessed, and exchanged. The computer industry is likely the most rapidly evolving sector in the history of humanity. Since the introduction of the personal computer, Gordon Moore, cofounder of Intel Corporation, observed that computer computational power doubles every 18 months. Unpredictable and powerful potentials are emerging from the parallel networking of computers in the World Wide Web. In a connected idea, it is estimated that the quantity of information is doubling every 24 months in the field of genetics, and in the field of biology, information is doubling every 5 years (Rifkin, 1998). Advances in MALDI-TOF MS have made femtomolar (10^{-15}) determinations common,

and scientific journals like Single Molecules (first issue in the year 2000) have emerged dedicated to reporting methods capable of detecting single atoms. These trends will likely continue to affect analytical technologies, and humanity will eventually acquire the ability to efficiently measure most chemicals in very low concentrations in any sample matrix.

Outlined below are the factors that the author predicts will markedly improve in the decades to come, based on trends already established for chemical analysis in research laboratories in instrumentation available commercially:

7.5.1. Miniaturization and Portability

The size of the analytical instruments, the quantity of reagents and sample required for analysis, will all continue to become smaller. The concept of miniaturization is intimately linked with speed of processing and ultimate sensitivity. Nanotechnology is currently the next frontier in miniaturization. Mass spectrometers are now becoming bench-top pieces of equipment, and will be seen commonly as hand held devices (Badman and Cooks, 2000).

7.5.2. Sensitivity and Accuracy

In the future, the ability to attain attomolar (atto = 10^{-18}) sensitivity, or measurement of fewer than a million molecules, will be common. Although the detection limit of one molecule has been achieved already (Badman and Cooks, 2000), technologically speaking, the routine ability to do so in drug residue analysis has a long way to go. Although these limits will be interpreted with caution and be suspect of accidental contamination, there is no reason to doubt the continuance of trends toward the limits of single molecule detection. The unequivocal identification of compounds in residue analysis is improving and will continue to improve, as mass spectrometry coupled with other separation techniques gains a greater role in routine residue analysis. These front-line MS systems are the best options for confirmatory identification of compounds, especially when they are coupled with specific filters like IAC, and coordinated with

improved computer spectral analysis programs and large and accessible MS residue fragment databases.

7.5.3. Analysis Efficiency

Analysis speed will continue to increase dramatically. The limiting factor in residue analysis will be sample processing and clean-up prior to quantification or detection: analyte detection, spectral acquisition, data processing and interpretation are not the bottle-neck. Advancement in robotics is lagging behind advancements in computer control; however, commercially available robotic systems are rapidly evolving for macromolecular sequencing (Gupta et al., 1999; Wasinger et al., 1995) and in the search for new drugs using combinatorial chemistry strategies (Schmatloch and Schubert, 2004). Separation technologies are rapidly being miniaturized and capable of high throughput, such as those contained on silicone chips (Ekstrom et al., 2002b; Little et al., 1997a; Nelson et al., 1997) (Figure 7.3) or microfluidic multiple target approaches on compact disc formats (Hsieh et al., 1998). These trends imply that residue analysis could be fully automated, and possibly contained into small cartridge modular concepts. Samples could be robotically applied to a cartridge in parallel arrays, with each cartridge performing a specific clean-up task, and each linked to another, until the final target probes are automatically loaded. Target probes, containing hundreds to thousands of samples, could be loaded as cartridge sets to the analyzer instrument. With high speed laser rates, and high speed, computer-controlled data acquisition and processing, systems such as a MALDI ion source coupled with MS/MS, could have unimaginable speeds of processing.

7.5.4. Affordability and Disposability

As with any material idea that has been mass produced in the industrial age, its cost to produce is reduced dramatically in the free market economic system if there is demand for it. The top-of-the-line computers have remained approximately the same cost over the last twenty years, yet their computing power has increased by thousands. Similarly, with the related technology of chemical analysis, the cost of current residue analysis capabilities will come down dramatically. State-of-the-art equipment may still

be relatively expensive, but would be capable of greater performance than current systems with respect to speed, sensitivity, and accuracy. Miniaturization and technologies such as the use of recombinant antibody fragments in residue extraction, will make the sample handling systems disposable, and will minimize the cost associated with solvents and their disposal. With the advent of large scale, routine food or environmental residue monitoring, there will be no need for initial screening systems like ELISA to identify positive samples for confirmation by a separate technology: front-line, confirmatory,

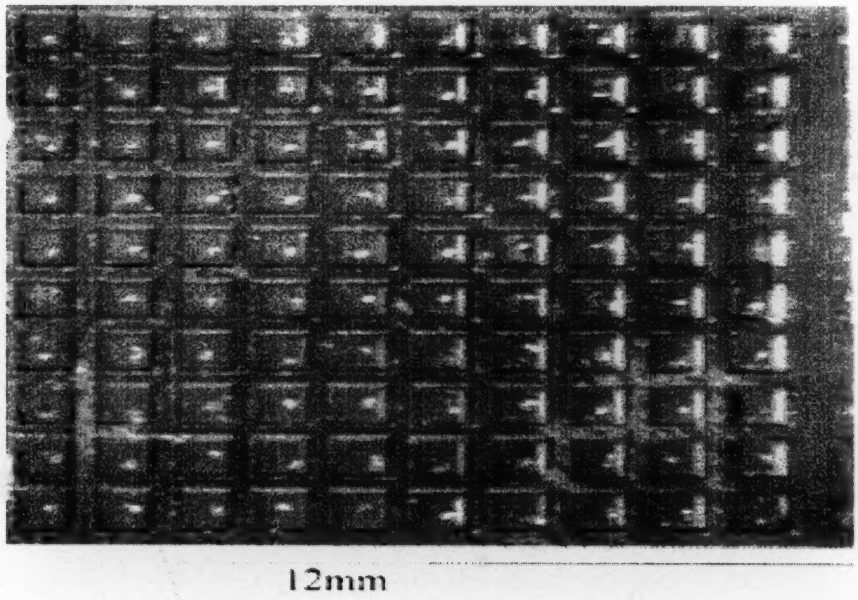

Figure 7.3. A 100 element array of miniaturized MALDI samples on a silicone chip. 6 nL matrix and 6 nL sample per well. (Adapted from Little et al., 1997).

high throughput analysis will be affordable. Disposability of clean-up systems will also improve the reliability of analysis due to decreasing the chances of contamination between samples.

7.5.5. Merging of Bioassay and Chemical Speciation Technologies

Bioassay systems have gained great utility in environmental residue analysis, not only because of their sensitivity, high throughput capability, and affordability, but more

importantly, because they monitor the integrated measurement of biological activity rather than concentration alone. To augment the information acquired by bioassay systems, chemical speciation is necessary to identify, understand and confirm modes of action of particular pollutants. Although currently these two important facets of residue analysis are separate, in the future they will likely be combined into one system. Classical toxicological models alone – examining the direct harmful effects of a compound to a cell or tissue - are not adequate now since trace level pollutants like dioxins have been linked to hormone disruption. New strategies to more efficiently combine bioactivity and chemical speciation will evolve. Initial sample clean-up may employ technologies such as recombinant antibodies that are capable of being specifically designed to mimic cellular or sub-cellular receptors, or enzymes, sensitive to compounds with hormone-like activity. Similar to strategies in combinatorial drug research, these clean-up strategies could screen environmental and food samples for compounds with potential hormonal activity and purify them. The purified compounds could then be immediately analyzed with high throughput MS systems, thus quickly enabling a correlation of chemical species' concentrations to potential biological activities.

7.6. PERSPECTIVES ON RISKS ASSOCIATED WITH CHEMICAL USE

7.6.1. A Sulfonamide-Specific Perspective

Over the last 50 years, many people have sustained large therapeutic exposures to sulfonamides, and it is also known that low ng/L level sulfonamide residues have been widely consumed by society in animal food products. Despite this long-term exposure of sulfonamides, and although 3% of the general population has an adverse reaction to these drugs (Tilles, 2001), there is no conclusive evidence that non-therapeutic sulfonamide exposure in food residues has caused adverse reactions. Except for perhaps penicillin (Borrie and Barrett, 1961a; Guillet et al., 2003; Moneret-Vautrin, 2003; Neugut et al., 2001; Schwartz and Sher, 1984; Siegel, 1959b; Wicher et al., 1969), the conclusive examples of adverse reactions due to drug contamination of food are rare. Sulfonamides are still routinely used in medicine as the drug of choice for acute, chronic, and recurrent

urinary tract infections. Since adverse reactions are reported to occur in as high as 20% of those who are therapeutically treated, it is possible the 3% figure for general population hypersensitivity could be explained by therapeutic treatment alone. However, if hypersensitivity to sulfonamides is linked to residues in food, researchers would best direct their efforts first to those individuals previously sensitized by therapeutic exposure.

Although very little information is available about the fate and impact of sulfonamides in the environment, their potential risks and consequences are linked to their role in causing and perpetuating multi-drug resistant bacteria, thus limiting their use in medicine. These risks are not measurable or determined at this time. The overall benefits of sulfonamides and antibiotics to prevent and treat human suffering in medicine have been remarkably positive, overall. However, unlike human medicine, the benefits of non-therapeutic sulfonamide use in agriculture are mainly economic. Although some argue that eliminating sulfonamides in animal feed increases animal suffering due to increased disease rates, most of this can be prevented by management adjustments, such as decreasing animal population density, increasing ventilation and living environment cleanliness, and ensuring access to clean water and food. Although the Swedish example points to an ability to adjust animal husbandry practices to adapt to the elimination of antibacterial agents and growth promoters in feed, it is unclear what the increased short-term costs in the price of meat, milk, and eggs would be in an unregulated, free market system. Even if the technical risks associated with agricultural use of sulfonamides could be measured, the central question at hand will always be more ethical in nature. The following question can be applied to all chemicals used in agriculture: "Do the benefits of using chemicals to increase the efficiency of food production outweigh the costs and risks?". The price of animal products may increase substantially without the use of sulfonamides, antibiotics, and growth promoters, so it is a matter of judgment to assess the benefits against the potential risks of food and environmental residues.

7.6.2. A General Organic Pollutant Perspective

Analytical chemistry is very important in both acquiring the data to initially assess risk associated with use of a chemical agent, but also later in monitoring food, environment, and living systems in determining and regulating individual and societal

risk levels. As technology improves, society will be better able to monitor chemicals in most samples. This will occur with confirmatory accuracy and precision, at ultra-low levels, and by small, portable devices that do not require high degrees of training to operate. It is conceivable that national and international governments could cooperate to establish a network of permanently installed residue monitoring stations around the world, sampling and analyzing the ecosystem on-site, and with real-time transmission of processed information back to central databases. It is very likely, that as sensitivity limits decline and trace-level residue analysis becomes affordable, most compounds will be found everywhere. What will society do with this information?

Information, by itself, is useless; individuals and society give information meaning by integrating it further into knowledge systems. Knowledge by itself has no meaning; individuals and society make value judgments about what is beneficial and what is not, what is sought and what is avoided. Knowledge has never been inherently good or bad. It is our value judgments that ascribe goals to our actions and generate the perception of benefit and risk. Every human activity has a relative risk associated with it, so it is important to place these risks in perspective. Risks can be established using several criteria based on statistical assessment of data. First, a chemical hazard is identified, then all available scientific data on dose-response relationships and exposure rates are assessed. Central to risk assessment, is defining the methods and assumptions pertaining to establishing a reference dose that, with a statistically defined confidence, harm or no harm will occur as a result of exposure to an agent. After a reference dose is determined (such as "no-observed-adverse-effect-level (NOAEL)"), then minimum risk safety levels can be estimated for a population by dividing the NOAEL by assigned adjustment factors associated with such things as population variability, extrapolation error from animal models, lack of chronic impact data or an incomplete database.

Another side of risk assessment, aside from determining the minimum residue limits for food or the environment, would be in the decision making process of whether to use or discontinue use of a chemical in agriculture, medicine, or industry. The precautionary principle is commonly used with regard to making policy decisions where inadequate information exists to make informed, scientific ones based on the assessment of toxicological and exposure research information. The precautionary principle is not a

process using the empirical scientific method, but rather relies upon logical guesses. It is used when the potential consequences are judged severe or irreversible, or expected benefits of using a chemical or technology do not outweigh the potential costs or negative impacts. Figure 7.4 is a flow chart that combines value judgments with scientific risk assessment to decide when to apply the precautionary principle.

7.6.3. Technology in Perspective: Solving the World's Problems

Regardless, whether using non-scientific precaution or the scientific method to assess risk, human judgment is always required in the end to arbitrarily assign a level of risk that is reasonable to take. It is important that scientists conduct their research in consideration of its moral implications, where they contemplate their actions with respect to a greater society and to the planet as a whole. In contrast to having a greater expected longevity than ever before, it is a disconcerting idea that soon individuals will have the capability to easily monitor and fixate on the toxins, carcinogens, hormone disrupters, and infectious pathogens in every breath, bite of food, and drink of water one takes. It is a sensational idea rife with the possibility to spawn many marketing strategies aimed at people's fears. It is a spectacle fully capable of distracting people from more important social considerations. It is the substance by which media will likely make more profit, as they shape people's values with little accountability to providing a balanced viewpoint. The onus is upon each scientist to choose carefully what he or she does, and to engage in communications - in consideration of the overall consequences - within scientific communities, government policy agencies, industries, and with the public at large.

Humanity is embarking on an unprecedented journey. The majority of world resources is accessed by those who live in the "developed nations", while the major proportion of humanity has not yet secured basic quality food, water, or shelter. And yet a large proportion the world's consumption is manifested as non-essential goods and services, transportation, healthcare and entertainment. The meanings of this growing disparity and paradox of consumption, and the subsequent environmental pollution caused by those who consume these resources, have more to do with value judgments such as who will control, benefit from, and be responsible for the material, information, energy, and genetic wealth of the planet.

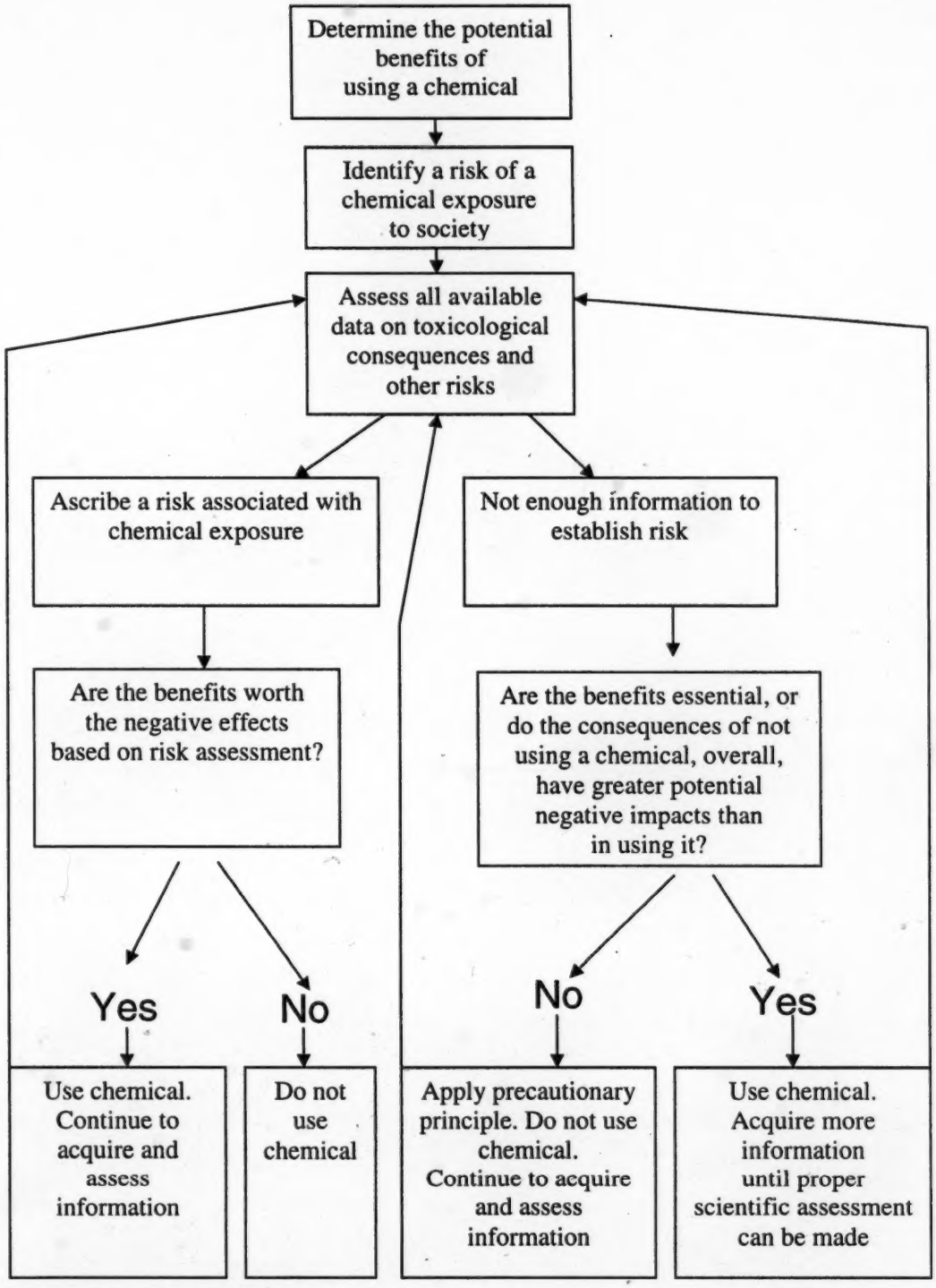
Determine the potential benefits of using a chemical
Identify a risk of a chemical exposure to society
Assess all available data on toxicological consequences and other risks
Ascribe a risk associated with chemical exposure
Not enough information to establish risk
Are the benefits worth the negative effects based on risk assessment?
Are the benefits essential, or do the consequences of not using a chemical, overall, have greater potential negative impacts than in using it?
Yes
No
No
Yes
Use chemical. Continue to acquire and assess information
Do not use chemical
Apply precautionary principle. Do not use chemical. Continue to acquire and assess information
Use chemical. Acquire more information until proper scientific assessment can be made

Figure 7.4. Decision-making flowchart in risk assessment combining scientific method and value judgment

In the developed world, there exists an irresponsible and ignorant reliance on technology to quickly fix crises, which in reality, require more complex, longer-term and fundamental changes to the structure and attitudes of society. It is important that people developing, managing, or consuming the advancements of science are aware that technology, alone, cannot solve the world's problems. Most problems are deeply rooted in the social and political realms at every level of society. When humanity finds a way to more fully embrace the responsibility and implications of its actions, it is then that technology will help it realize a physical security for most of the world, and to fully engage the possibilities of the mind.

Chapter 8

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Chapter 9

APPENDICES

9.1. INTRODUCTION

This chapter represents a large amount of the preliminary results and discussion regarding the development of antibodies and immunoaffinity columns, and is presented as a set of appendices so the interested reader can examine these concepts in more detail. Most of this information was absent from the body of the text, to avoid over-emphasizing relevant subtleties and complexities at the expense of an uninterrupted flow of ideas.

9.2. DEVELOPMENT OF ANTIBODIES

9.2.1 Rabbit Immunization

Sulfonamides, sulfamethazine (SMT) or sulfathiazole (STZ), were covalently linked to LPH with an azo-linker arm or succinyl-linker arm, making 4 different immunogens used to immunize a total of 10 rabbits. Although the thesis goal was to develop a trace residue detection system for SMT, a STZ system was also investigated so that better generalizations could be made about the variability in performance of sulfonamide IAC systems. Rabbits were immunized in three separate batches using SMT or STZ conjugated to *Limulus polyphemus* hemolymph (LPH) (Table 9.1).

Table 9.1. Batch designation and sulfonamide immunogen used for 10 rabbits

Batch	Sulfonamide immunogen[1]			
	SMT-azo-LPH	**STZ-azo-LPH**	**SMT-succinyl-LPH**	**STZ-succinyl-LPH**
Batch 1	1A, 1B	1C, 1D		
Batch 2	2A, 2B	2C, 2D		
Batch 3			3A	3B

[1]Sulfonamide-protein conjugates are described with the following abbreviations: sulfamethazine = SMT; sulfathiazole = STZ; LPH = *Limulus polyphemus* hemolymph protein; azo = azo linkage; succinyl = succinyl linkage.

Batches 1 and 2 represent eight rabbits immunized with SMT or STZ linked to LPH by the diazo reaction (Figure 2.1). Batch 3 immunizations were performed using STZ-LPH and SMT-LPH conjugates linked by a N^4-succinyl linkage so that the resulting antibodies could be used to further evaluate the linker arm effects that have been noted to cause problems in previous IAC research involving haptens (Ben Rejeb et al., 1998a).

The immunogen design was considerate of making an IAC system capable of equally extracting a parent sulfonamide and its respective N^4-acetyl metabolite. These acetyl metabolites represent the main form excreted from many animals, including pigs (Mitchell et al., 1986; Nouws et al., 1986a), and is important to measure in tracking the fate of sulfonamides from agricultural sources into the environment. All sulfonamide antibacterial compounds have the same N^4-amino-benzene end in common, and are differentiated only by the functional group substituted onto the N^1-amino of the basic sulfonamide structure (refer to Figure 1.2 of Chapter 1). The aromatic N^4-amino end of the sulfonamide molecule was chosen as the site to conjugate to LPH in making an immunogen, thus exposing the end unique to each sulfonamide, and not the end in common with both the parent sulfonamide and its acetyl metabolite. The resulting immunogen would therefore theoretically produce specific antibodies capable of recognizing both the parent compound and the acetylated metabolite, yet differentiating them from other sulfonamides (see Figure 3.2 of Chapter 3).

Table 9.2 shows the sulfonamide molar incorporation rates into protein conjugates synthesized for immunization (LPH conjugates) and for coating ELISA plates or making sulfonamide IAC systems to purify antibodies (bovine serum albumin (BSA) conjugates). Moderate sulfonamide incorporation rates were achieved (approximately 10-30 moles sulfonamide/ mole protein), based on values previously reported (Garden and Sporns, 1994). The first succinyl-linked conjugates made (labeled in Table 2.2 as the sulfonamide-succinyl-protein-1 conjugate groups) were not soluble in water, likely because excessive protein cross-linking was catalyzed by too much of the dehydrating reagent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). Although these LPH gels were adequate for making emulsions for immunization, the corresponding insoluble BSA gels could not be used for coating ELISA plates. The conjugation procedures were altered by decreasing the EDC added, to achieve soluble BSA conjugates (sulfonamide-

Table 9.2. Molar incorporation rates[1] of sulfonamides into protein conjugates[2] (moles of sulfonamide/moles of protein)

SMT-azo-BSA	9.1
STZ-azo-BSA	9.4
SMT-azo-LPH	19.6
STZ-azo-LPH	29.1
SMT-succinyl-BSA-1	23.5
STZ-succinyl-BSA-1	9.76
SMT-succinyl-BSA-2	12.4
STZ-succinyl-BSA-2	10.2
SMT-succinyl-LPH-1	13.4
STZ-succinyl-LPH-1	17.0

[1]Molar incorporation rates were determined using Equations 2.1 and 2.2.

[2] Sulfonamide-protein conjugates are described with the following abbreviations: sulfamethazine = SMT; sulfathiazole = STZ; LPH = *Limulus polyphemus* hemolymph protein; BSA = bovine serum albumin; azo = azo linkage; succinyl = succinyl linkage. Hyphenated attachment of numbers 1 or 2 to some conjugates refer to two different procedures used to link succinyl derivatives to BSA or LPH, as described in Chapter 2, section 2.4.1.

succinyl-BSA-2) that could be used as coating antigen for ELISA without sacrificing molar incorporation rate.

The titre of each rabbit serum is reported in Table 9.3, and was determined by serially diluting each one to achieve an absorbance of approximately 1.5 based on standard ELISA Format 1 described in the experimental methods. Rabbits immunized with azo-linked conjugates produced sera with high titres and specificity as reported previously (Assil et al., 1992b; Garden and Sporns, 1994; Sheth and Sporns, 1991), however, much lower titres were achieved for sera from rabbits immunized with the succinyl conjugates. High affinity antibodies are generally required to achieve sensitive ELISAs and make IACs capable of extracting haptens (Delaunay et al., 2000). Average polyclonal antibody-specific affinity and their concentration in the serum affect the titre, and both factors are important in amassing enough specific antibodies to make an anti-hapten IAC system.

The sera from the N^4-azo immunogen system had the highest titres and were selected as the best candidates to select antibodies for IAC. However, titres of the azo-linked and the succinyl-linked immunogen systems could not be used to evaluate their relative immunogenicity because of a confounding mistake. The succinyl-linked immunogens were not injected into the appropriate rabbit for the first immunization of batch 3 rabbits, 3A and 3B [1]. This was the only logical way to explain an anomaly in the trend of post-immunization titres over time, where sera had relatively higher titres to the sulfonamide to which they supposedly had not been exposed. Similar results were later confirmed using plates coated either sulfonamide-succinyl-BSA or sulfonamide-azo-BSA, thus the discrepancy could not be explained by an antigen-coating effect. Some cross reactivity is always expected of an antibody to a set of related haptens, but it is unreasonable to expect a better titre to a hapten-protein conjugate system that has a structurally different epitope exposed during immunization. A mistake in the initial immunization may also explain the low titres for batch 3 sera against either SMT-BSA or STZ-BSA antigens, because the proper booster immunizations would not have

[1] Analysis of post-immunization blood samples determined that rabbit 3A was likely immunized incorrectly with STZ immunogen, and rabbit 3B was immunized incorrectly with SMT immunogen. Later immunizations were correct as intended (3A getting SMT and 3B getting STZ), because thereafter they had significant titres for both SMT and STZ. As a result, both types of specific antibody could be immunopurifed from each rabbit.

Table 9.3. Antibody titres[1]

Antibody	Immunizing antigen	Titre against SMT-azo-BSA 6 ng/well	Titre against STZ-azo-BSA 6 ng/well
1A	SMT-azo-LPH	1/250k	-
1B	SMT-azo-LPH	1/250k	-
1C	STZ-azo-LPH	-	1/125k
1D	STZ-azo-LPH	-	1/100k
2A	SMT-azo-LPH	1/200k	-
2B	SMT-azo-LPH	1/75k	-
2C	STZ-azo-LPH	1/75k	-
2D	STZ-azo-LPH	1/75k	-
3B	STZ-succinyl-LPH	1/32	1/4k
3A	SMT-succinyl-LPH	1/2k	1/16

[1] The titre of a serum or antibody solution was defined as the dilution required to achieve an A_{450} of 1.5 in ELISA Format 1 as described in the experimental section. Sulfamethazine- (SMT) or sulfathiazole- (STZ) azo-linked conjugates with bovine serum albumin (BSA) were used to coat the plates.

followed the important first immunizations using "Freund's complete" adjuvant. Regardless of these irregular results, both batch 3 rabbits were immununopurified for antibodies with specificity for both sulfonamide types, and then later used to examine linker arm effects as was the original intention.

9.2.2. Purification of Antibodies

To produce a high capacity anti-sulfonamide IAC system it was necessary to purify the sulfonamide-specific antibodies from non-specific antibodies, before attaching the specific fraction to a solid phase IAC support. This enrichment process could theoretically increase the sulfonamide capacity of an anti-sulfonamide IAC by approximately ten fold over the more common method of attaching unenriched antibodies. A ten fold enrichment was based on the assumption that 10-15% of serum antibodies are sulfonamide-specific (Delaunay et al., 2000; Hennion and Pichon, 2003).

First, protein G affinity chromatography (for batch 1 sera) and ammonium sulfate precipitation (for batch 2 and 3 sera) were used to separate antibodies from other components in rabbit sera, based on well established procedures (Harlow and Lane, 1988). This step purified both specific and non-specific immunoglobins from these sera relative to their ELISA specificity (Format 1) to the corresponding sulfonamides used in the immunogens. Next, the sulfonamide-specific fraction of the purified antibodies was separated for each sera. This was achieved using immunoaffinity enrichment columns made by immobilizing an appropriate sulfonamide-BSA conjugate onto a CNBr-activated Sepharose 4B solid phase (STZ-azo-BSA conjugate for anti-STZ antibody purification and SMT-azo-BSA conjugate for anti-SMT antibody purification). These columns functioned by immunopurification principles, were a form of IAC, and purified the antibody by binding an immobilized sulfonamide hapten. Later, these purified antibodies would be immobilized onto the same type of solid phase medium, and this subsequent IAC form would function to purify the sulfonamide hapten.

In the process of separating sulfonamide-specific antibodies from non-specific antibodies, these enrichment columns were washed free of non-specific proteins and the antibodies were eluted with acidic buffer. The protein content and anti-sulfonamide ELISA activity were monitored for the first two rabbit sera processed (1B and 1D) to

account for the sulfonamide-specific fraction in all steps of the purification. The elution buffer conditions used for this IAC system (1 M acetic acid, pH 2.5) resulted in approximately 100% accounting of the original immunoglobin protein added to the enrichment columns based on UV spectrometry (Table 9.4). Since the immunoglobin was accounted for, it was assumed that negligible immunoglobin was still bound to the enrichment columns. Therefore, the amount of antibody protein recovered in the pooled acidic eluants from the sulfonamide-azo-BSA IAC was considered to be the main sulfonamide-specific fraction of the polyclonal immunoglobin population (17% and 26% for 1B and 1D, respectively).

Approximately 50-60 mL serum was obtained per rabbit obtained by single cardiac puncture procedures before sacrificing each rabbit. With a total yield of about 200-400 mg antibody (specific and non-specific) as determined by UV spectrophotometry (approximately 5 mg per mL serum). A detailed accounting for the recovery of specific antibodies was not done for all sera; however the approximate yield for the sulfonamide-specific fraction was estimated for each antibody (Table 9.5). Batch 1 sera had significantly higher ($p<0.05$) specific antibody yields per mL serum (1.13 ± 0.11 mg/mL , n=4) or per rabbit (57 ± 9 mg, n=4) compared to the other batches of sera (0.32 ± 0.04 mg/mL, n=6, and 18 ± 2 mg, n=6, on a serum and rabbit basis, respectively, for batch 2 and 3 values combined).

ELISA analysis of the protein G and sulfonamide-BSA enrichment column purifications also determined that the anti-sulfonamide antibody activity had been conserved in the eluants with negligible anti-sulfonamide activity remaining in the flow-through fractions (Table 9.6). The titre of any fraction from antibody purification could be determined by ELISA Format 1 (see Chapter 3). This was the basis for determining relative antibody activity among fractions, and for the accounting of specific sulfonamide activity during antibody purification relative to the original total activity contained in the unpurified serum. ELISA analysis was an important complement to UV spectrometry, establishing that there was no significant loss of antibody binding activity in the purification process. Sulfonamide-BSA columns were used approximately ten times in the project, and no appreciable loss in their binding capacity was observed.

Table 9.4. Total immunoglobin protein[1] from sulfonamide-azo-BSA affinity column[2] purification of two different sera

	Immunoglobins from 1B (anti-SMT-azo-LPH[3])	Immunoglobins from 1D (anti-STZ-azo-LPH[3])
A) Total antibody added to sulfonamide-column (mg)	61.1 mg	52.6 mg
B) Total antibody not specific to sulfonamide (mg) (as total protein from final flow-through of sulfonamide-column)	47.7 mg	37.3 mg
C) Total anti-sulfonamide antibody (mg) (as total protein pooled acid eluants from sulfonamide-column)	10.5 mg	13.4 mg
Percent of total antibody represented as sulfonamide-specific (100*C/A)	17.2 %	25.5 %
Percent of antibodies accounted for from IAC (100*(B + C)/A)	95.3 %	96.4 %

[1]Antibody determination using UV spectrometry.

[2] Sulfonamide was conjugated to bovine serum albumin (BSA) protein by an azo linkage, and this conjugate was covalently attached to a solid phase support to make an immunoaffinity column capable of purifying anti-sulfonamide antibodies.

[3] Antibody abbreviations refer to the corresponding sulfonamide-protein immunogens used to immunize the rabbit. SMT=sulfamethazine, STZ=sulfathiazole; LPH= *Limulus polyphemus* hemolymph; azo=azo linker arm.

Table 9.5. Yield of sulfonamide-specific antibody from rabbit serum

Antibody ID	Specificity to sulfamethazine (SMT) or to sulfathiazole (STZ)	Immunogen linker arm system	Sulfonamide-specific antibody yield [1] (mg/ mL serum)	Approximate total yield of specific antibody per rabbit[2] (mg)
1A	SMT	azo	1.16	70
1B	SMT	azo	0.88	35
1C	STZ	azo	1.06	53
1D	STZ	azo	1.40	70
2A	SMT	azo	0.29	16
2B	SMT	azo	0.17	9.4
2C	STZ	azo	0.37	20
2D	STZ	azo	0.49	27
3A	STZ, SMT[2]	succinyl	0.19, 0.10[3]	10, 6[3]
3B	SMT, STZ[2]	succinyl	0.16, 0.15[3]	9, 8[3]

[1] The antibody protein yield was determined with UV spectrometry (λ = 280 nm; extinction coefficient of 1.35 mL/mg*cm).

[2] Sulfonamide-specific antibody yields were approximated by multiplying the specific antibody concentration (mg/ mL serum) by the estimated total volume of serum harvested from each rabbit.

[3] Based on ELISA analysis of post-immunization blood samples, it was determined that rabbit 3A was immunized incorrectly with STZ immunogen, and rabbit 3B was immunized incorrectly with SMT immunogen. Later immunizations were likely correct as intended (3A getting SMT and 3B getting STZ), because thereafter they had significant titres for both SMT and STZ. As a result, both types of specific antibody could be immunopurifed from each rabbit.

Table 9.6. ELISA evaluation of anti-sulfonamide activity during Protein G and enrichment column purification[1] of anti-SMT and anti-STZ sera

Fraction of Serum Sample Purified through Protein G or sulfonamide-BSA affinity chromatography	% ELISA response relative to original serum response[2]	% standard error of the mean (n=2)
1B Serum (from rabbit immunized with SMT-azo-LPH conjugate)		
original serum	100.0	13.2
protein G flow-through	0.1	0.2
SMT-BSA affinity column flow-through	2.8	0.2
SMT-BSA affinity column elution	111.7	0.9
1D Serum (from rabbit immunized with STZ-azo-LPH conjugate)		
original serum	100.0	13.2
protein G flow-through	0.1	0.3
STZ-BSA affinity column flow-through	3.4	0.3
STZ-BSA affinity column elution	124.7	4.5

[1] Protein G and/or immunoaffinity columns (made with sulfamethazine (SMT) or sulfathiazole (STZ) conjugates with bovine serum albumin (BSA)), where used to purify antibodies from serum.

[2] Flow through or eluant fractions of Protein G or sulfonamide-BSA affinity column purifications were analyzed with ELISA Format 1 to account for antibodies. Serum was diluted by 50 k and purification fractions were diluted equivalently relative to the respective serum dilution used.

When determining the titres for polyclonal sera, there were high background reactions to LPH or BSA protein controls bound onto ELISA plates, especially for lower dilutions of antibody and for higher plating concentrations of the protein controls. This was expected for LPH antigen, because it was the protein used to make immunogens. However, it was not expected for BSA, because it is unrelated to LPH in species origin and had not been used to immunize the rabbits. However, in testing these sera using competitive ELISA (sections 3.3.1, 3.3.2, and 3.3.3), they did not have significant specific activity to the BSA component of the sulfonamide-BSA conjugate coating the plate. It is likely that a sulfonamide-coated BSA conjugate is not antigenically comparable to pure BSA because of the sulfonamide conjugation to it. In other words, the BSA epitopes that the non-sulfonamide-specific antibodies in sera IB and 1D were recognizing, were not available for binding in the sulfonamide-BSA conjugate.

In response to these observations of antibody cross reactivity to pure protein antigens in ELISA, an experiment was conducted using anti-sulfonamide-azo-LPH sera 1B and 1D to determine if immunopurifying sera reduced this cross reaction to pure BSA. Antibodies were evaluated by ELISA Format 2 (see Chapter 2, section 2.7.3), which was designed to test non-specific binding. It was found that immunopurification of either 1B or 1D sera virtually eliminated their responses to pure LPH and BSA unconjugated proteins bound it ELISA plates, without loss of response to the corresponding sulfonamide-protein conjugates coated on the plate (see Figure 9.1). This means that, in contrast to the ELISA the immunopurification process eliminated any doubt about having problems in ELISA with cross reaction to protein antigens.

9.2.3. Effect of Antibody Immunopurification on ELISA Specificity to Sulfonamide Derivatives

Another competitive ELISA experiment was performed in parallel to *ELISA Experiment 1: Evaluation of a Linker arm Effect Using Indirect Competitive ELISA by Varying the Competitor Ligand Derivative* reported in Chapter 3, section 3.3.1. This experiment evaluated batches 1 and 3 serum and their respective sulfonamide-specific immunopurified antibodies to demonstrate if immunopurification affected relative

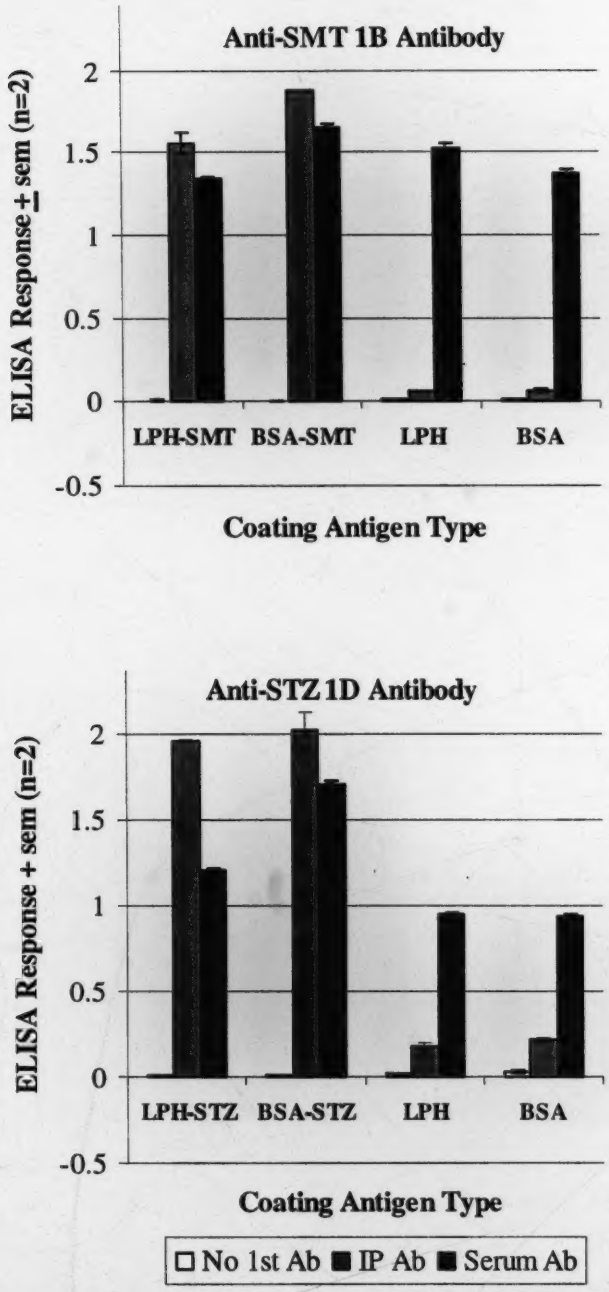


Figure 9.1. Comparison of ELISA response (ELISA Format 2) of immunopurified antibodies (IP Ab), serum antibodies (Serum Ab), and a "no 1st antibody" blank, for both anti-sulfamethazine (anti-SMT at top) and anti-sulfathiazole specificities (anti-STZ at bottom), to four coating antigen types; LPH-sulfonamide, BSA-sulfonamide, LPH, and BSA. The "No 1st antibody blank" was a negative control using diluent buffer alone instead of antibody. Where the error bars are not visible they are smaller than the symbol.

antibody specificity among sulfonamide derivatives. Changes in IC_{50} among sulfonamide derivatives in competitive ELISA were compared for both immunopurified antibodies and serum antibodies diluted to achieve standard conditions using ELISA Format 3 (Figure 9.2). The relative pattern of changes in average IC_{50} values by derivative type varied dramatically for the different antibody sources. But when considering the overall average effect of immunopurification within either batch for all sulfonamide derivatives collectively, there was no particular pattern or significance ($p>0.05$, $n=4$) of immunopurifcation. There was an overall trend towards the reduction of IC_{50} values, but this change was not significant at the 95% confidence level. When considering different sulfonamide derivative treatments within each batch group separately, the change in relative affinity after immunopurification increased (IC50 decreased) for the parent sulfonamide competitors for batch 1 antibodies ($-28.3 \pm 8.0\%$, $n=4$; $p< 0.05$). Although it had been shown that for two sera (1A and 1D) the enrichment column immunopurification process conserved the anti-sulfonamide specific activity (Table 9.6), it is possible that this enrichment process was capable of removing some of the higher affinity antibodies exhibiting a strong linker arm effect. As mentioned in Chapter 2, a common problem sited in using serum immunopurification to enrich hapten-specific antibodies, is the loss of high affinity antibodies. It is possible that the highest affinity antibodies do not elute under conditions mild enough to not destroy the antibodies. With respect to the relative distribution of antibody specificities, this could have shifted the overall antibody affinity towards becoming more sensitive (i.e. lowering IC_{50}) to the parent sulfonamide in the remaining population. It may be possible to decrease the linker arm effect more dramatically by selectively eluting only lower affinity antibodies from a sulfonamide-linker arm-BSA immunopurification column first. The higher affinity antibodies, which are more specific to the sulfonamide-linker arm of the immunopurification column, could
be selectively retained if milder elution conditions were used in the enrichment column system (e.g. pH 4).

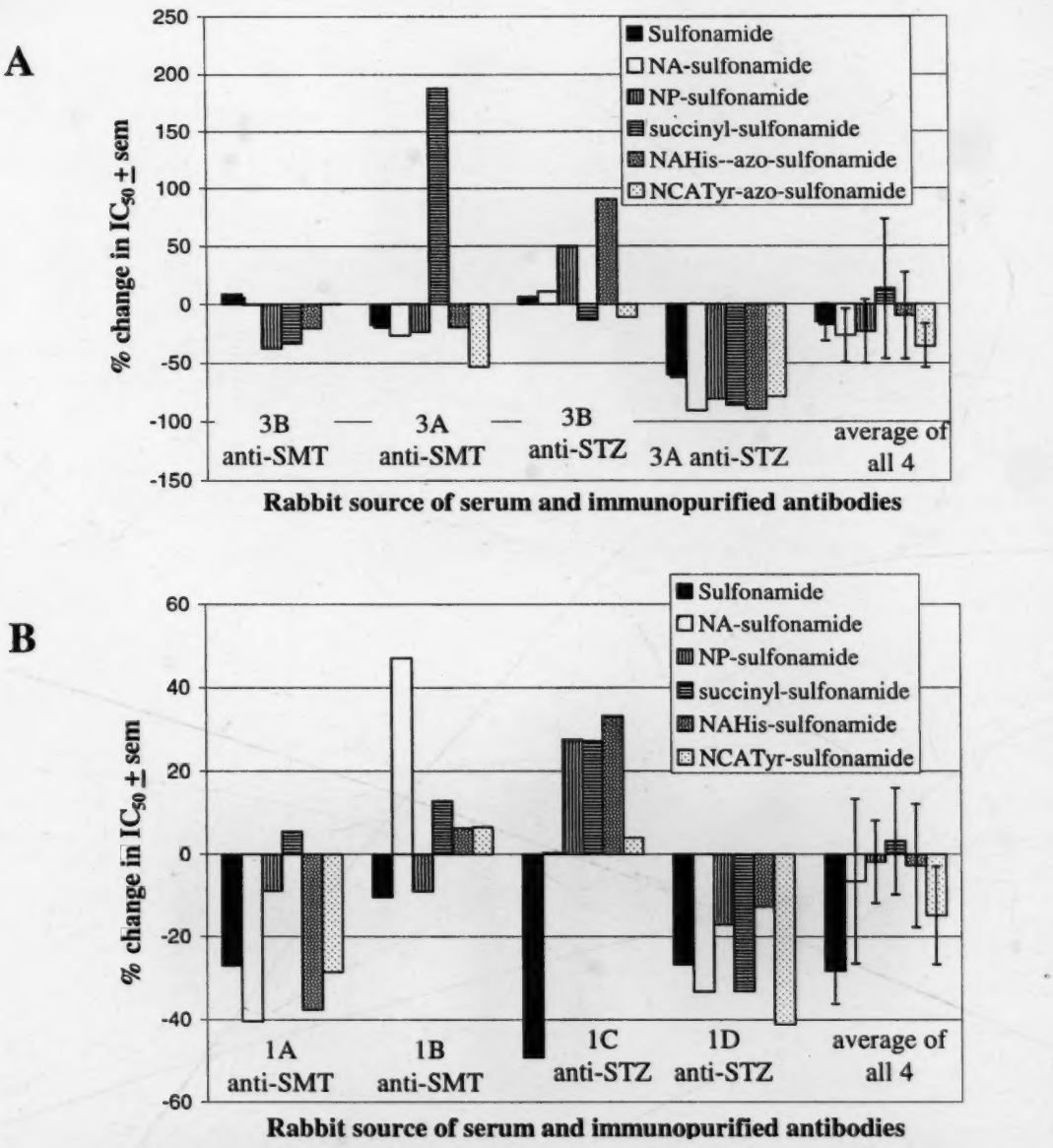


Figure 9.2. Effect of immunopurification on IC_{50} values for sulfonamide derivatives in competitive ELISA. **A**=batch 1 antibodies of the N^4-azo linker immunogen system. **B**=batch 3 antibodies of the N^4-succinyl linker immunogen system. The effect of immunopurification was measured for different sulfonamide derivatives represented by different patterned bars. Each bar represents the % change in IC_{50} in competitive ELISA (Format 3) under standard conditions. For a given competitor and antibody (Ab), the % change in IC_{50} was calculated as 100*((IC_{50} with immunopurifed Ab) – (IC_{50} with serum))/ (IC_{50} with serum). Negative change means increase in assay sensitivity. The average change in IC_{50} in competitive ELISA following immunopurification for all 4 sera within a batch is shown at the right of each figure ± standard error of the mean.

9.3. INITIAL IAC DEVELOPMENT EXPERIMENTS

9.3.1. Establishing Elution Conditions

The original acidic conditions (1 M acetate buffer, pH 2.5) used to elute and immunopurify antibodies from sulfonamide-BSA antibody enrichment columns also worked for eluting sulfonamides from these antibodies immobilized on IAC. However, the simplest, most non-hazardous and non-destructive elution system was desired for eluting sulfonamides from IACs. Many elution conditions (varying pH, [NaCl], and ethanol or methanol content) were tried in seeking to have the lowest organic solvent content and the pH closest to neutrality (to minimize damage to the antibodies), and lowest salt concentration (to minimize the interference for MALDI-TOF MS analysis). IACs made with immunopurified antibodies 1A and 1D were used to establish a standard elution buffer of 10 mM HCl (pH 2.5). This system was capable of eluting more than 95% of the parent sulfonamide and its N^4-acetyl metabolite derivative with 4 mL of elution buffer for a 0.4 mL immunopurified antibody IAC. These relatively mild conditions (10 mM HCl) were applied for the first two IAC experiments of Chapter 4 (sections 4.2.1 and 4.2.2), and all SPIE MALDI-TOF MS analysis of environmental samples using "1B" IAC reported in chapters 5 and 6. However, it was discovered that incomplete elution occurred for sulfonamide derivatives (e.g. NA-SMT or NAHIs-SMT) from the anti-SMT "1A" IAC using the 10 mM HCl elution conditions (reported in Chapter 4, section 4.2.3). This problem was corrected by adding ethanol to the 10 mM HCl buffer at 20% by volume.

9.3.2. Theoretical Considerations Determining IAC Capacity

The next important consideration was to assess the theoretical capacity of an anti-SMT IAC to see if it was reasonable that it could hold 1μg of SMT (1 ng/L x 1 L = 1 μg). The assumptions in this estimate were as follows:

1. The immunoglobin purified from rabbit serum is 100% pure IgG and has a molecular weight of 150 kDa.
2. Antibody is purified so that all of the IgG is specific for SMT.
3. Each antibody molecule binds two SMT molecules.

4. Every specific antibody attached to the IAC solid phase support is available for binding SMT.

5. During the synthesis of the IAC, the Sepharose gel is capable of covalently binding approximately 0.007 g of antibody per 1 mL of hydrated column (Pharmacia Biotech, Uppsala, Sweden).

6. Recovery is 75% (a reasonable figure reported commonly in the literature for IAC (Delaunay et al., 2000)).

7. Functional capacity allowance factor: the column must be 10 X greater than required to bind 1μg SMT to give extra allowance for binding of other metabolites and internal standards without competition, and to compensate for functional loss of column after repeated use.

Equation 9.1 then describes the minimum volume of an IAC required to bind 1 μg of SMT based on the assumptions listed above for an IAC system constructed of polyclonal IgG purified for the SMT-specific fraction.

Equation 9.1. *Volume IAC/ 1μg SMT (mL/μg)* = (150,000 g /mol IgG x 10^{-6} g SMT) ÷ (278 g / mol SMT x 2 mol SMT/ mol IgG x 0.007 g IgG/mL IAC x 0.75 recovery rate x 1/10 *functional capacity allowance factor*) = 0.5 mL

Normally at least 3 or 4 column volumes are necessary to fully (95%) elute a compound from an IAC. This would imply that a 1 μg SMT sample extracted by a 0.5 mL IAC would elute into about a 2 mL volume, giving a SMT concentration of 500 ng/L in the eluant, which is the detection limit for MALDI-TOF MS for these compounds. Having theoretically established that a sufficiently small volume IAC was capable of extracting 1 μg SMT detectable by MALDI-TOF MS, experiments were conducted to develop and evaluate IACs for sulfonamides.

9.3.3. IAC Antibody Incorporation Rates and IAC Sulfonamide Capacities

Using CNBr-activated Sepharose is one of the easiest and most common ways of linking a protein to a solid phase support (Figure 9.3). Initial investigations focused on this particular method for immobilizing antibodies. The first rabbit sera available for the

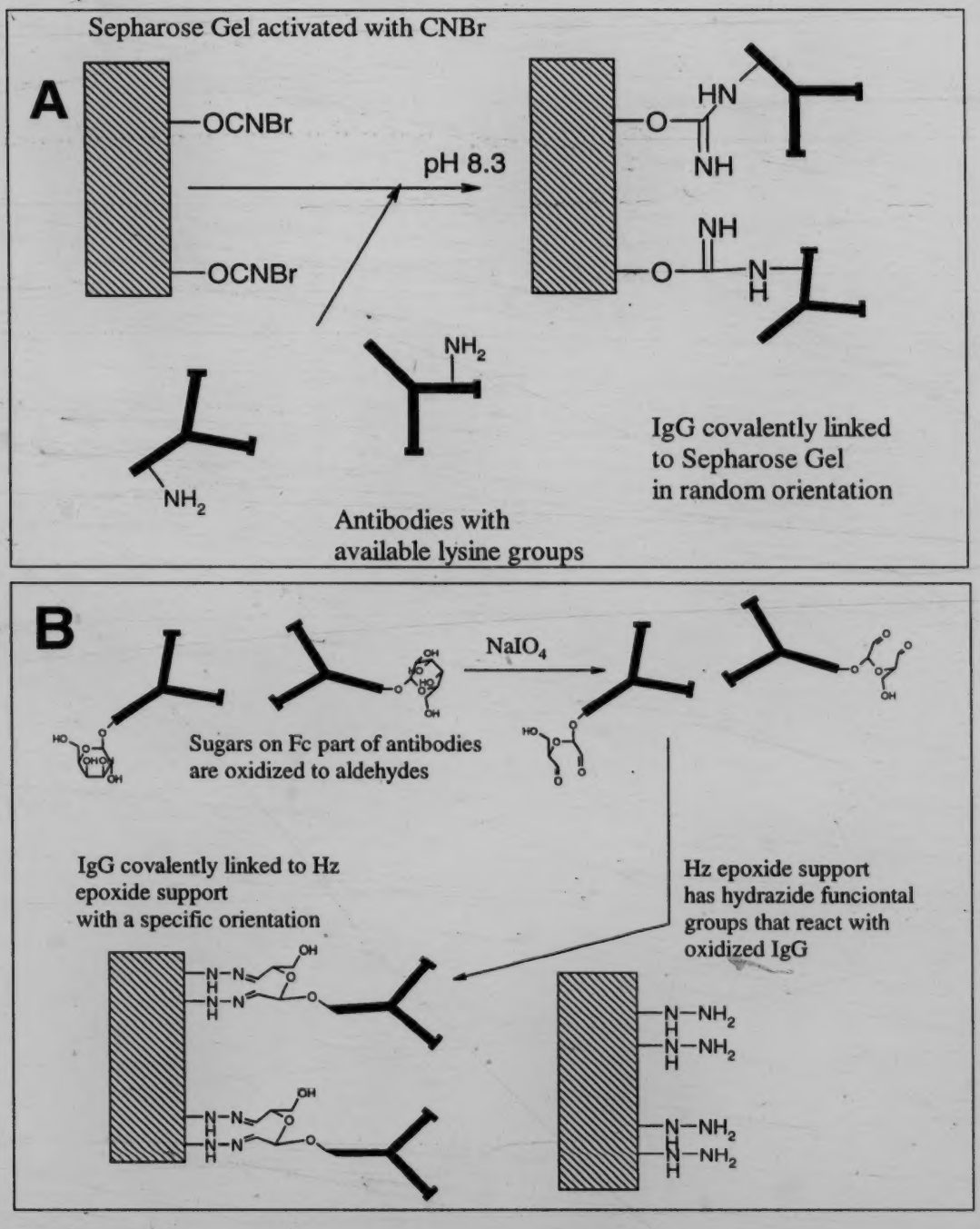


Figure 9.3. Schematic diagrams showing the coupling and hypothetical antibody orientation for covalently binding antibody (Y shaped object) to CNBr-activated Sepharose solid support (A) or to a hydrazine activated epoxide solid support (B).

initial IAC work were from the batch 1 immunizations. Table 9.7 reports the sulfonamide capacities of several IACs made with these antibodies that were either Protein G-purified alone, or were then enriched further for the sulfonamide-specific fraction using sulfonamide-BSA affinity chromatography.

It was important to maximize IAC capacity because this is often a limiting factor in the practical performance of a hapten IAC. In order to determine the maximum practical sulfonamide IAC capacity possible, higher antibody incorporation rates were attempted than recommended by the manufacturer (recommendation of no higher than 7 mg antibody/mL gel). All antibody incorporation rates for CNBr-activated Sepharose supports were 95-99% for 10 mg or less of antibody reacted per mL gel. However, when more than 10 mg of antibody was mixed per mL activated gel, antibody incorporation saturation dynamics began to occur as demonstrated by lower percent incorporation rates (data not shown). The manufacturer of the gel (Pharmacia Biotech, Uppsala, Sweden), warns that steric limitations are often a problem in immunoaffinity chromatography, where antibody incorporation rates of greater than 7 mg IgG per mL hydrated gel can cause diminished column extraction capacity for the antigen per mg of antibody incorporated. The rationale for this phenomenon is that antibodies attached to IAC become too crowded, and the antigens are not afforded adequate access to the binding sites.

The IAC capacities are represented relative to the theoretical maximum in Table 9.7 and are based on Equation 2.4 (see Chapter 2, section 2.4.2). The same information is graphed in Figure 9.4, which shows a positive linear correlation between IAC capacity as a function of IAC antibody incorporation rates. The overall immunopurifed IAC performance was 80% of the theoretical maximum, which was determined by the quotient of the function's slope divided by the corresponding slope representing maximum theoretical IAC capacity. The IAC binding capacities containing immunopurifed antibodies were exceptional for a polyclonal anti-hapten IgG IAC, especially when one considers there was no control over orientation of IgG attachment to the solid phase with CNBr binding, and also because one expects some denaturation and breakage of IgG during handling. Sulfonamide capacities were directly related to the mass of specific antibody that had been incorporated with no sign of the function

Table 9.7. Antibody incorporation rates and sulfonamide capacities of individual preparations of immunoaffinity gels (CNBr-activated Sepharose 4B) by antibody source and purification method

Antibody Source[1]	Hapten type: Sulfamethazine (SMT) or Sulfathiazole (STZ)	Purification system[2]	Antibody incorporated into IAC[3] *mg antibody per mL hydrated gel*	IAC sulfonamide capacity[4] *sulfonamide (nmol) per mL hydrated gel*	IAC sulfonamide capacity[5] *% theoretical maximum*
1B	SMT	G	6.0	23	6.3
1B	SMT	G	11.8	34	9.4
1B	SMT	G and IP	5.9	47	60
1B	SMT	G and IP	10.3	95	69
1A	SMT	AS and IP	17.7	173	73
1D	STZ	G	6.0	29	7.4
1D	STZ	G	11.7	39	10
1D	STZ	G and IP	5.1	50	73
1D	STZ	G and IP	9.3	103	83
1D	STZ	G and IP	11.1	90	61
1C	STZ	AS and IP	15.2	220	109

[1] All batch 1 antibodies were made with an N4-azo type immunogen. Antibody sera from different rabbits of this batch are represented by a different letter: 1A, 1B, 1C, or 1D.

[2] G = Protein G affinity chromatography; IP = immunoaffinity purification by sulfonamide-BSA enrichment column; AS = ammonium sulfate precipitation.

[3] By Equation 2.3a.

[4] By Equation 2.5.

[5] By Equation 2.4

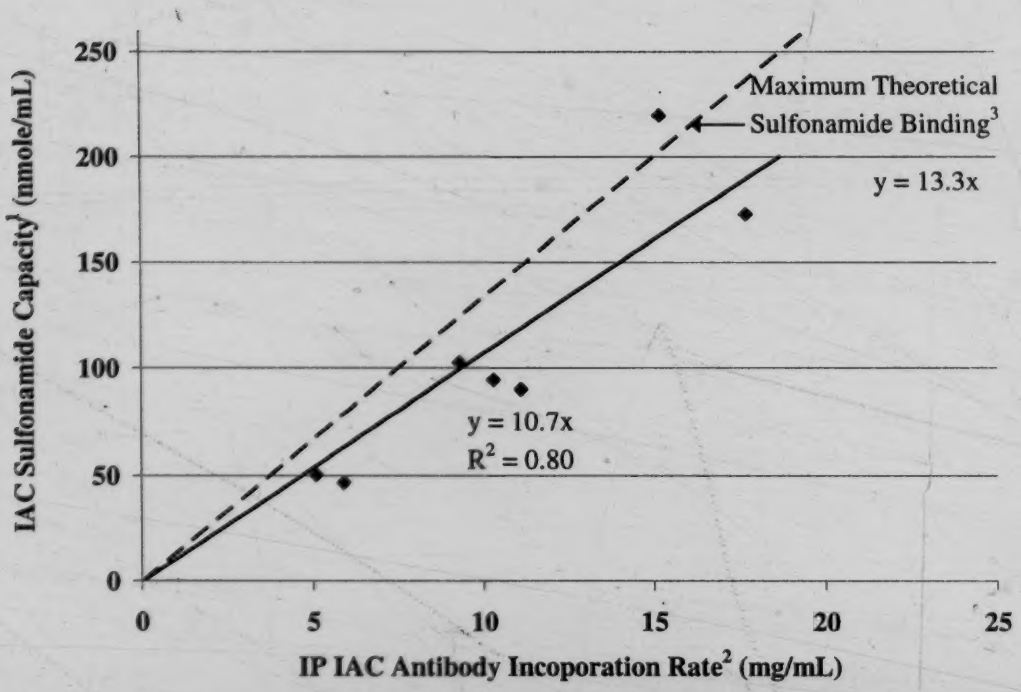


Figure 9.4. CNBr-activated Sepharose immunoaffinity column (IAC) sulfonamide capacity as a function the incorporation rates of immunopurifed antibodies (IP) into IAC.

[1] IAC sulfonamide capacities were determined using Equation 2.5. SMT- (n=3) and STZ- (n=4) specific IACs were used to create this graph.

[2] IP IAC antibody incorporations rates were determined by Equation 2.3.

[3] The theoretical capacities of IAC columns were determined by Equation 2.4.

reaching a plateau (Figure 9.4). This means there was no evidence for steric inhibition of IAC sulfonamide binding with greater incorporation rates of 7 mg immunopurified antibody/ mL IAC gel. Steric inhibition of IAC binding of larger protein antigens is a reasonable expectation for high IAC incorporation rates (above 7 mg antibody/mL IAC gel), but the smaller size of the sulfonamide likely made this effect negligible.

The average sulfonamide IAC loading capacity made with unenriched 1B and 1D antibodies (Protein G and ammonium sulfate–purified) was 8.3 $\pm$ 1.2% (n=4) of the theoretical maximum. Similar poor capacities have recently been reported by others in the development of polyclonal anti-sulfonamide IACs for clean-up of biological samples (Crabbe et al., 1999; Li et al., 2000). These teams used Protein G or ammonium sulfate-purified antibodies to achieve high antibody incorporation rates into CNBr-activated gels (8.9 and 9.3 mg IgG/mL gel, respectively), yet their IAC loading capacities were 5.8% and 6.7% of the theoretical maximum (1.9 µg of SMT, and 2.0 g of STZ, respectively). Conversely, IACs made with immunopurifed anti-sulfonamide antibodies were able to achieve sulfonamide capacities circa 50 µg sulfonamide/ mL gel. These immunopurifed IACs represent a ten fold improvement in theoretical binding capacity compared to the corresponding unenriched IACs made with conventional procedures with IgG not immunopurified. This means the size of an anti-sulfonamide IAC can be reduced for a given application, thus accommodating potential efficiency gains through miniaturization and reducing elution volumes.

There is another 1.5 fold capacity gain possible by using Fab antibody fragments instead of whole IgG (MW=150 kDa). Fab fragments contain the antibody variable binding domains, which can be enzymatically cleaved using papain and then purified from the Fc portion (50 kDa) to produce two 50 kDa hapten-binding subunits (Harlow and Lane, 1988). Alternatively, if using recombinant antibody fragments single chain variable fragments (ScFv) that constitute just the variable region of the antibody binding site (25 kDa), an almost 3 fold capacity gain is possible compared to whole cloned IgG molecules. Further gains in loading capacity could be achieved by using just the variable portion of the light or heavy antibody chain, which are one half the size of ScFv. It has been shown that some monoclonal antibodies only use one of the variable domains (V_H) in binding a small hapten (Murata et al., 2002), and camels have a naturally occurring

single-domain heavy chain (Spinelli et al., 2001). In either case, if V_H antibodies were obtained against a drug, they could be used to result in a 12 fold increase in loading capacity over the analogous IgG molecule. Assuming 20 mg fully functioning antibody protein/mL CNBr-activated Sepharose is the upper incorporation limit possible, and steric and orientation effects for binding sulfonamides are negligible, the upper limit for IAC using antibody V_H fragments is about 200-400 μg of SMT/mL gel, or more than 100 fold higher than conventional methodologies with unenriched polyclonal antibodies.

9.3.4. CNBr-Activated Sepharose IAC Antibody "Bleed"

A common problem reported with the CNBr activated Sepharose IAC system is that the covalent bond attaching the antibodies is not stable (Hock et al., 1995). Consequently, antibodies can be lost from the column, which is referred to as antibody "bleed". The rate of bleed is not usually significant enough to affect the column capacity or longevity compared to other factors that impact the activity of the bound antibodies, such as destructive elution conditions. An experiment was designed using an IAC made of 1D immunopurified antibodies to see if the bleed phenomenon existed under the chosen conditions. A freshly prepared IAC was conditioned as normal, with three alternating washings of 0.1 M Tris pH 8 + 0.5 M NaCl and 0.1 M acetate buffer pH 4 + 0.5 M NaCl, to remove antibodies that were not covalently attached. The column was then subjected to a series of phosphate-buffered saline (PBS) and acid/NaCl washings to approximate the normal operation of the column. Washings were collected, diluted by 10 fold with PBS with 0.05% Tween (PBST), and analyzed in ELISA using Format 1 (Table 9.8). The IAC antibody bleed rate was determined using standard ELISA Format 1, and was based on the direct relationship between the antibody concentration and the ELISA response. The positive control antibody dilution was established at limiting concentration (1/60 k), so that the antibody concentration tested would be directly proportional to the A_{450}.

Absorbance readings greater than blank indicated that antibody bleed was occurring, although at very low levels. The highest bleed rate was 161 ng/mL, which represented about 1.6×10^{-3}% of the column's antibodies per mL of washing. Even if the IAC sustained this bleed rate, it would take over 6 L of washing to deplete 10% of the

Table 9.8. ELISA Detection of anti-STZ antibodies "bleeding" from an Anti-STZ immunoaffinity column [1]

Controls or washings applied to STZ-BSA coated ELISA wells	A_{450}	Antibody Bleed Rate (ng/mL)[2]
Controls in PBST		
1D anti-STZ-azo-LPH antiserum 1/60k	1.76 ± 0.02	-
Blank	0.15 ± 0.00	-
Washings diluted by 10 in PBST (applied in order as listed)		
PBS rinse 1	1.28 ± 0.02	161
PBS rinse 2	0.64 ± 0.00	70
Acid-NaCl rinse 1	0.60 ± 0.04	64
Acid-NaCl rinse 2	0.19 ± 0.00	6
PBS rinse 3	0.72 ± 0.01	81

[1] A 0.4 mL affinity purified anti-sulfathiazole (STZ) immunoaffinity column was made using immunopurifed antibodies 1D and conditioned using the standard protocol. PBS or acid rinse (0.1 M sodium acetate pH 2.7 + 0.5 M NaCl) solutions were applied to the column in 2.5 mL volumes consecutively, and then the corresponding fractions that flowed through the column were collected, diluted by 10 using PBST, and 100 µL aliquots were tested using ELISA Format 1.

[2] Bleed rate was calculated based on Equation 2.8.

antibody. This bleed rate would be significant for a direct, one-step IAC clean-up of 1 L samples required for trace residue analysis if the columns were regenerated and reused. However, with low volume sample clean-up with this IAC system, there would likely be little to no impact of antibody bleeding on the IAC performance over time as it is regenerated and reused. Some IAC columns were later reused in low volume environmental sample clean-up over 100 times without noticeable change in column capacity.

Yet there are other implications of interference if this IAC were used for sample clean-up prior to competitive ELISA quantification of sulfonamides as has been reported for sulfamethazine in urine (Crabbe et al., 1999). The bleeding antibodies would confound competitive ELISA results, where low concentrations of limiting antibody are necessary to establish a dynamic response. The antibody bleed would significantly contribute to ELISA response if samples eluted from IAC were applied at low dilutions to an ELISA plate. A CNBr-activated Sepharose IAC system therefore cannot be recommended to clean-up samples prior to competitive ELISA unless this bleed issue is addressed by both diluting samples adequately and running the necessary controls to monitor bleed effects.

9.3.5. CNBr-Activated Sepharose IAC "Breakout"

"Breakout" is a phenomenon of any solid phase extraction (SPE) system where the ligand of interest is washed out of the solid phase by shear mass action of the sample volume. Breakout can occur in any SPE system, and is a measure of the affinity that the solid phase has for a particular ligand under a given solvent conditions (Delaunay et al., 2000). The interaction between the solid phase and the ligand can be non-specific, as with the hydrophobic interaction of C_{18} columns for SMT, or can be specific, as with the antibody-antigen interaction of anti-SMT IAC for SMT. Regardless of the nature of the interaction, the degree to which the equilibrium is shifted toward the bound state (i.e. higher binding constant), is the degree to which the breakout phenomenon is less pronounced. Preliminary work in IAC development established that the recovery of sulfonamides from a 1 L volume was about ¼ of that compared to a 10 mL loading volume. The breakout volume was defined as the volume of water required to diminish

the sulfonamide bound on a previously sulfonamide-saturated column by one half compared to a normal 10 mL water washing. The column was saturated with analyte using a 10 mL sample (exceeding at least twice the column's saturation capacity), washed with a variable volume of water (from 10 mL to 1L), eluted, and then the sulfonamide was quantified in the eluant colorimetrically with the Bratton-Marshall (BM) Assay. The breakout point for immunopurified 1B IACs was occurring between 100 mL and 200 mL.

This finding early in the method development was critical in determining that the anti-SMT IAC could not be practically used in a one step process for trace-level sulfonamide enrichment. A primary step was necessary to achieve the sulfonamide concentration goals from a large enough volume of aqueous sample (1 L) containing trace amounts of sulfonamides (1 ng/L) to obtain adequate sulfonamide (1 μg) for MALDI-TOF MS detection.

9.3.6. CNBr-Activated Sepharose IAC Collapse

Another obstacle in the quest to efficiently extract ng/L levels of sulfonamides from 1 L of water, was that the Sepharose 4 B solid phase support could not withstand the sustained hydrostatic pressures required to achieve loading rates of more than 1 mL/min. Shortly after the initiation of column loading, the support matrix would collapse and the column would stop flowing after the application of 100 mL of sample. Since anti SMT-IAC had problems with both break out and column collapse when extracting large volume samples, it was decided that an additional high-recovery, non-specific SPE concentration step was necessary for sulfonamide concentration prior to IAC. If post SPE sample volumes were kept below 50 mL to avoid IAC break out, bleed, or collapse problems, an efficient and effective IAC purification step could then follow SPE concentration.

9.3.7. Development of AffiPrep Hz™ Hydrazide Support-IAC

An attempt was made to address the issue of breakout and column collapse by using a more physically robust support. AffiPrep Hz Hydrazide (Hz support) was chosen to evaluate and is a commercial product for linking proteins onto a methyl methacrylate epoxide polymer. This support can withstand higher hydrostatic pressures than

Sepharose. The hydrazine functional groups of the support react with the aldehydes of oxidized antibody sugar moieties (Figure 9.3). Since the hydrazine system binds antibodies to the solid phase via the Fc portion, the antibody binding sites are oriented away from the solid phase support, theoretically making them available for interacting with antigen.

Compared to the CNBr-activated Sepharose support, antibody incorporation rates into the hydrazide support were less than one half (45% and 36% for anti-SMT and anti-STZ IACs, respectively). To determine the binding capacity of the columns, the Hz support IACs were conditioned, and then challenged to saturating conditions of sulfonamide. The columns were then washed with 5 mL water, and the total sulfonamide was accounted for in flow-through and elution fractions. Similar tests were simultaneously applied to analogous CNBr-activated Sepharose IACs.

Except for the anti-STZ IAC Hz support, all sulfonamides were accounted for in in the flow through and acid elution fractions of IACs (Table 9.9). Almost one half of the STZ added to the anti-STZ IAC was not accounted for in these first two fractions, but instead was eluted and accounted for only after DMSO was applied. Except for the anti-STZ IAC Hz support, the sulfonamide capacities for all IACs were close to or less than 100% of the theoretical maximum based on the immunopurified antibody incorporation rates (Equation 2.4). On the other hand, the anti-STZ IAC Hz support system retained STZ at 320% of its theoretical maximum binding capacity (32.2 vs. 10.1 μg expected). It was thought that this support was binding STZ non-specifically, a conclusion that was supported by the fact that DMSO was required for full elution of STZ from the IAC. The same antibody used in CNBr-activated Sepharose required only the normal acidic eluant to fully elute STZ. Although the Hz support was able to withstand higher hydrostatic pressures without collapsing and was reported to have a more stable covalent linking system for the antibody, its potential for binding non-specifically prevented it from being a viable candidate for sulfonamide IAC.

Compared to the Hz support system, the CNBr-activated Sepharose system was determined to be the best compromise for sulfonamide extraction due to its ability to make IACs with high hapten binding capacity and low non-specific binding. Despite the disadvantages associated with the CNBr-activated Sepharose columns (sulfonamide

Table 9.9. Accounting of sulfonamides loaded at saturation levels to Affiprep Hz™ Hydrazine and to CNBr-activated Sepharose 4B IAC supports in flow through and elution fractions

Sulfonamide specificity and column type[1]	Sulfonamide[2] loading to IAC (μg)	A Flow-thru (μg)	B Acid elution (μg)	C DMSO elution (μg)	D Theoretical maximum capacity[3] (μg)	Percentage accounted in all fractions A+B+C based on D
Anti-STZ Hz	30	4.4	17.3	14.9	10.1	122%
Anti-SMT Hz	30	23.7	7.5	0.7	8.0	106%
Anti-STZ CNBr	40	20.3	19.2	0.0	17.6	99%
Anti-SMT CNBr	40	20.9	22.4	0.0	30.7	108%

[1] Immunopurifed antibodies 1A and 1C were used for anti-SMT and anti-STZ IACs, respectively, and CNBr activated Sepharose supports (CNBr) and AffiPrep Hz Hydrazide support (Hz) were the solid phase supports used to covalently immobilize the antibodies.

[2] SMT was used to apply to anti-SMT columns and STZ was used to apply to anti-STZ columns.

[3] 100% of theoretical binding capacity was determined by Equation 2.4 based on antibody incorporation rates.

Equation 2.4: Theoretical maximum IAC sulfonamide binding (nmol)

$$= \frac{(\text{IgG incorporation rate into IAC in mg/mL}) * (2 \text{ sulfonamide binding sites/IgG}) * 10^6}{\text{IgG MW (150 kDa)}}$$

breakout with large sample volumes, antibody bleed, and column collapse), these problems were overcome by the development of the coordinated solid phase immunoextraction (SPIE) system, employing both non-specific SPE and immunoextraction of sulfonamides (reported in Chapter 5).

9.4. COMPETITIVE ELISA FUNCTIONS OF CHAPTER 3, SECTION 3.3.1

The appendices in this chapter represent a series of ELISA competitions using Format 3 (see Chapter 2, section 2.7.4) which were discussed Chapter 3 (section 3.3.1). Twenty different competition series were performed using antibodies from ten rabbits immunized with the sulfonamides, sulfamethazine (SMT) or sulfathiazole (STM), which were conjugated to *Limulus polyphemus* hemolymph (LPH) by either an azo or a succinyl linkage. In the appendices, the antisera raised against LPH sulfonamide conjugates with a given linkage are referred to by the following nomenclature: "anti-sulfonamide-azo-LPH" for antisera from the azo conjugate linkage immunization system, and "anti-sulfonamide-succinyl-LPH" for antisera from the succinyl conjugate linkage immunization system. The rabbit sera were either tested in their serum form or in their sulfonamide-specific immunopurifed form (see experimental section 7.3.3 for immunopurification procedure).

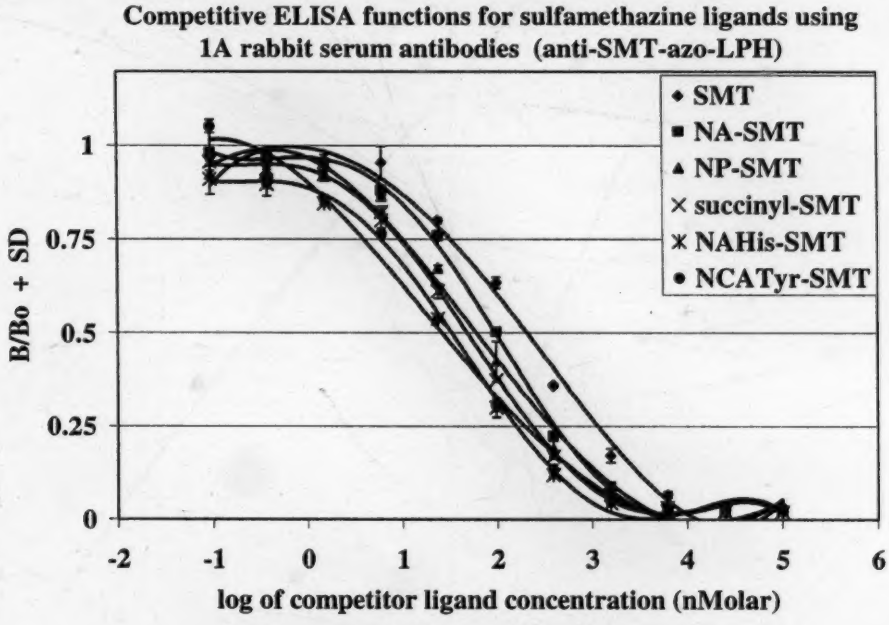
In each competition, a limiting antibody concentration was challenged to bind either the sulfonamide-azo-bovine serum albumen (sulfonamide-azo-BSA) antigen coated to the ELISA plate at a constant amount, or to one of 6 different sulfonamide derivatives at various concentrations in solution. SMT-azo-BSA and STZ-azo-BSA conjugates were used to coat plates for competitions involving anti-SMT and anti-STZ antibodies, respectively. Abbreviations used for the soluble sulfonamide derivatives tested in anti-SMT antibody competitions were sulfamethazine (SMT), N^4-acetylsulfamethazine (NA-SMT), N^4-propionylsulfamethazine (NP-SMT), N^4-succinylsulfamethazine (succinyl-SMT), N^4(N-chloroacetyl-L-tyrosine)sulfamethazine (NCATyr-SMT), and N^4(N-α−acetyl-L-histidine)sulfamethazine (NAHis-SMT). Abbreviations used for the soluble sulfonamide derivatives tested in anti-STZ antibody competitions were sulfathiazole (STZ), N^4-acetyl sulfathiazole (NA-STZ), N^4-propionyl

sulfathiazole (NP-STZ), N^4-succinyl sulfathiazole (succinyl-STZ), N^4(N-chloroacetyl-L-tyrosine) sulfathiazole (NCATyr-STZ), and N^4(N-α−acetyl-L-histidine) sulfathiazole (NAHis-STZ).

Each competition series (shown one per page) is displayed both graphically, as a collection of functions, and in tables, documenting the competition by reporting the corresponding IC_{50} (± standard error of the mean (sem); n=2) and cross reactivity values of the functions. The functions describe the competitions as the relationship between B/B_o and the concentration of a competing sulfonamide derivative. ("B" and "B_o" are defined as the ELISA absorbencies of a given competition and the no-competition blank, respectively). B/B_o values for hapten competitions were determined by comparing a competition ELISA response (B) to the blank ELISA response (B_o; using PBST + 1% BSA) to give normalized and visually comparable B/B_o ratios. ELISA IC_{50} was defined as the concentration of competing free sulfonamide required to inhibit by 50% a limiting amount of antibody from binding the solid-phase sulfonamide-BSA antigen coated to the plate. Antibody cross reactivity to a related ligand was then defined as a function of IC_{50}. Percent antibody cross reactivity = 100*A/B, where A = IC_{50} reference sulfonamide (SMT or STZ), and B = IC_{50} sulfonamide competitor (*Equation 2.10.*).

A remarkable variety was found in both the IC_{50} values for parent sulfonamides for different antibodies, and also in the relative patterns of cross reactivity for the sulfonamide derivatives within each antibody competition. The complete competitive ELISA curve functions represent an extensive amount of data not manifested in the corresponding IC_{50} and cross reactivity values. As a collection of functions, they offer an overall impression for subtleties, in both the variety and trends, which are not obvious from single IC_{50} values alone.

Appendix 9.4.1. Competitive ELISA with Anti-SMT Serum 1A



Sulfonamide derivatives in competitive ELISA using IA serum antibody (anti SMT-azo-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)	±	sem		% cross reactivity
SMT	200	±	2	a	100
NA-SMT	102	±	5	b	195
NP-SMT	67.8	±	5.3	c	294
succinyl-SMT	47.2	±	0.7	d	423
NAHis-azo-SMT	35.2	±	1.9	e	567
NCATyr-azo-SMT	33.4	±	0.5	e	597

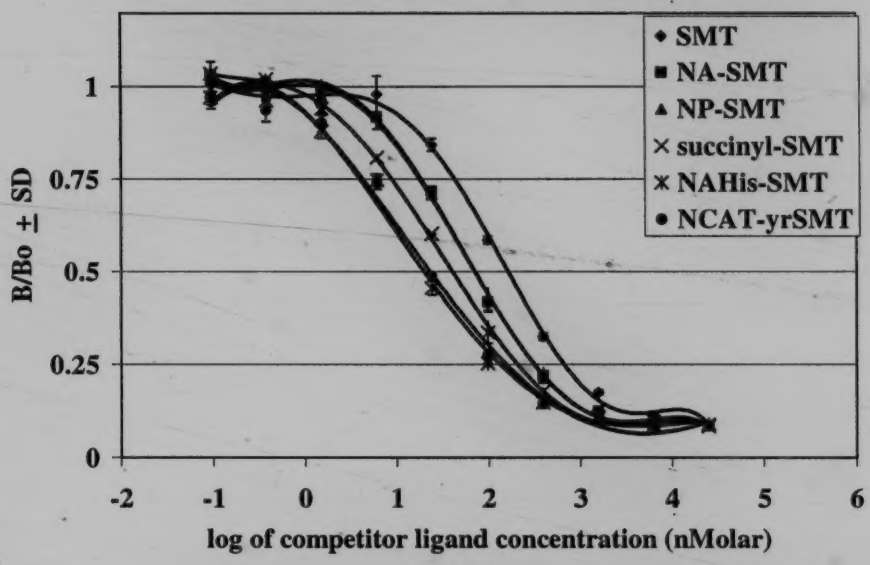
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.2. Competitive ELISA with Anti-SMT Immunopurified Antibody 1A

Competitive ELISA functions for sulfamethazine ligands using 1A rabbit immunopurified antibodies (anti-SMT-azo-LPH)



Sulfonamide derivatives in competitive ELISA using 1A immunopurified antibody (anti SMT-azo-LPH) and SMT-azo-BSA coating antigen

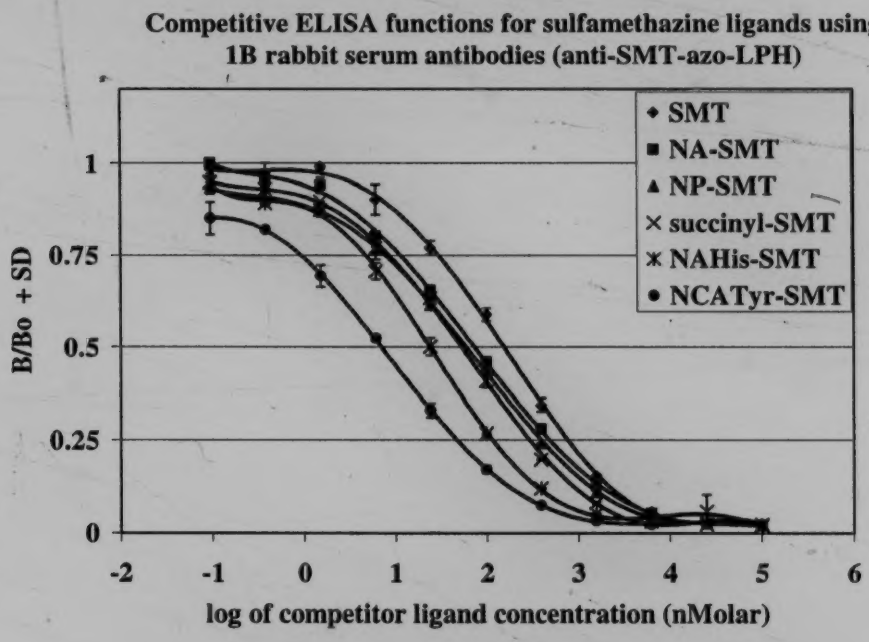
Competitor	IC_{50} (nM) Mean (n=2)		sem		% cross reactivity
SMT	146	±	2	a	100
NA-SMT	61.1	±	3.6	b	239
NP-SMT	61.8	±	4.7	b	236
succinyl-SMT	49.8	±	3.2	b	293
NAHis-azo-SMT	21.9	±	1.4	c	665
NCATyr-azo-SMT	23.9	±	0.3	c	611

IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.3. Competitive ELISA with Anti-SMT Serum 1B



Sulfonamide derivatives in competitive ELISA using IB serum antibody (anti SMT-azo-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)	±	sem		% cross reactivity
SMT	148	±	14	a	100
NA-SMT	70.1	±	2.1	b	211
NP-SMT	69.7	±	0.9	b	212
succinyl-SMT	57.0	±	4.9	b	260
NAHis-azo-SMT	21.7	±	2.7	c	683
NCATyr-azo-SMT	8.07	±	0.35	c	1835

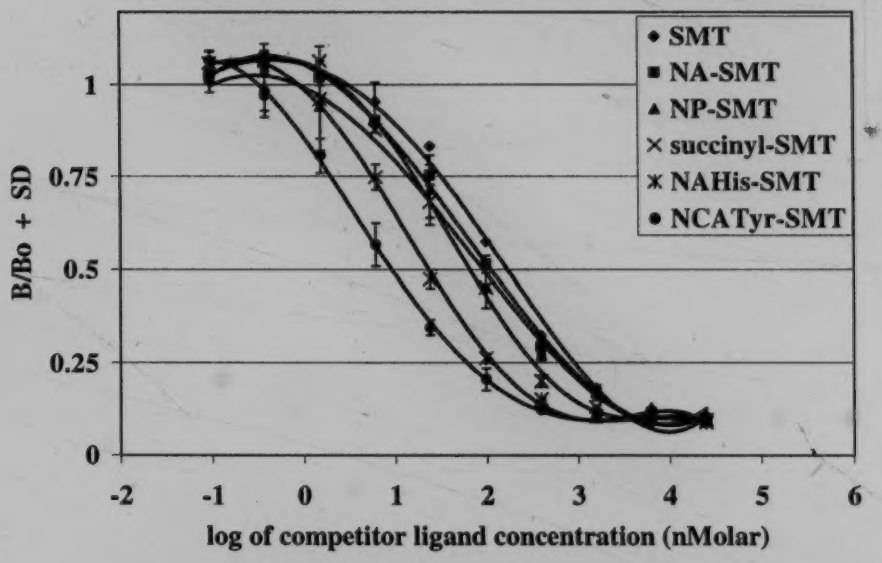
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.4. Competitive ELISA with Anti-SMT Immunopurified Antibody 1B

Competitive ELISA functions for sulfamethazine ligands using 1B rabbit immunopurified antibodies (anti-SMT-azo-LPH)



Sulfonamide derivatives in competitive ELISA using 1B immunopurified antibody (anti SMT-azo-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)	±	sem		% cross reactivity
SMT	133	±	3	a	100
NA-SMT	103	±	2	b	129
NP-SMT	63.4	±	7.4	c	209
succinyl-SMT	64.4	±	3.3	c	206
NAHis-azo-SMT	23.0	±	1.2	d	577
NCATyr-azo-SMT	8.59	±	0.43	e	1545

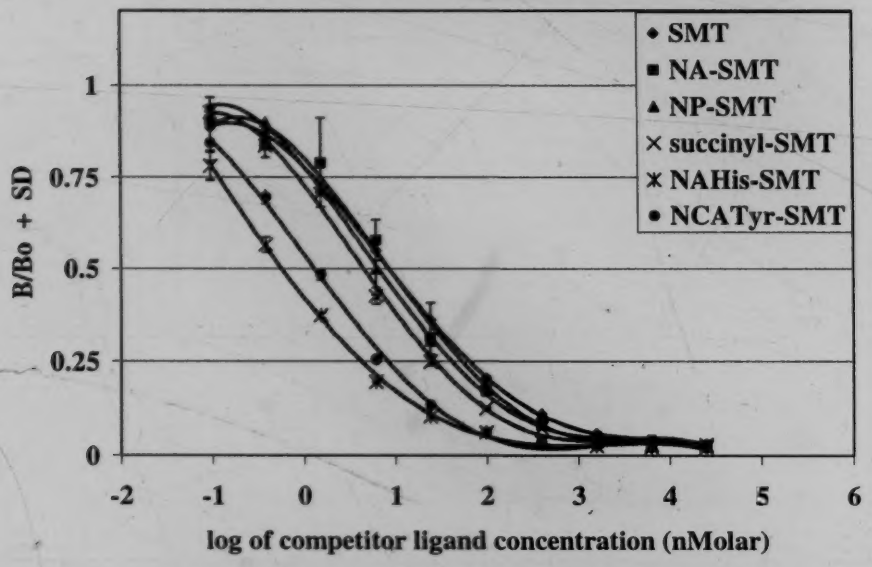
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.5. Competitive ELISA with Anti-SMT Serum 2A

Competitive ELISA functions for sulfamethazine ligands using 2A rabbit serum antibodies (anti-SMT-azo-LPH)



Sulfonamide derivatives in competitive ELISA using 2A serum antibody (anti SMT-azo-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)		sem		% cross reactivity
SMT	8.97	±	0.72	a	100
NA-SMT	7.44	±	0.24	ab	121
NP-SMT	6.10	±	1.73	ab	147
succinyl-SMT	4.39	±	0.38	b	204
NAHis-azo-SMT	0.595	±	0.057	c	1508
NCATyr-azo-SMT	1.31	±	0.02	c	683

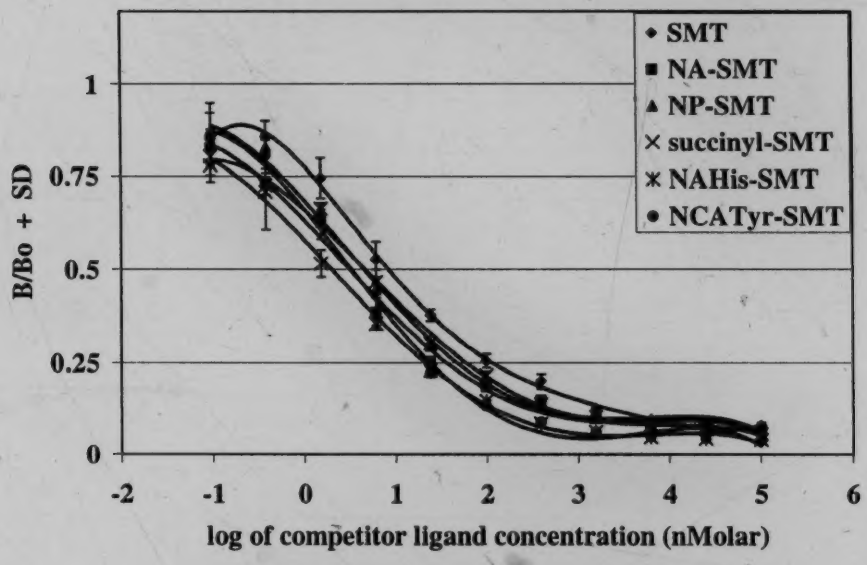
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.6. Competitive ELISA with Anti-SMT Serum 2B

Competitive ELISA functions for sulfamethazine ligands using 2B rabbit serum antibodies (anti-SMT-azo-LPH)



Sulfonamide derivatives in competitive ELISA using 2B serum antibody (anti SMT-azo-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)		sem		% cross reactivity
SMT	8.13	±	1.84	a	100
NA-SMT	4.02	±	0.14	b	202
NP-SMT	3.76	±	0.45	b	216
succinyl-SMT	3.64	±	0.40	b	223
NAHis-azo-SMT	1.38	±	0.25	b	588
NCATyr-azo-SMT	2.86	±	0.40	b	284

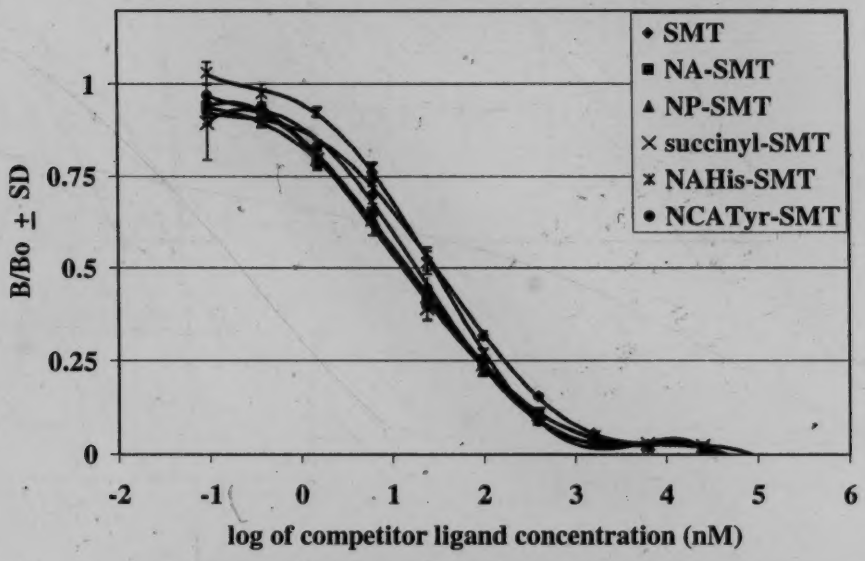
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.7. Competitive ELISA with Anti-SMT Serum 3A

Competitive ELISA functions for sulfamethazine ligands using 3A rabbit serum antibodies (anti-SMT-succinyl-LPH)



Sulfonamide derivatives in competitive ELISA using 3A serum antibody (anti SMT-succinyl-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)		sem		% cross reactivity
SMT	32.2	±	3.1	a	100
NA-SMT	15.6	±	1.1	cd	207
NP-SMT	14.8	±	1.6	cd	218
succinyl-SMT	12.4	±	0.8	d	260
NAHis-azo-SMT	26.5	±	0.4	b	122
NCATyr-azo-SMT	21.4	±	0.3	bc	151

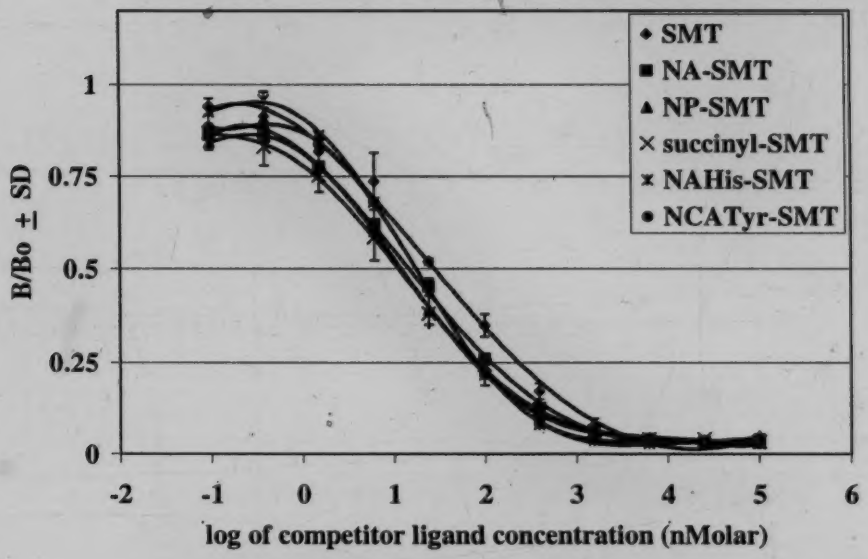
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.8. Competitive ELISA with Anti-SMT Immunopurified Antibody 3A

Competitive ELISA functions for sulfamethazine ligands using 3A rabbit immunopurified antibodies (anti-SMT-succinyl-LPH)



Sulfonamide derivatives in competitive ELISA using 3A immunopurified antibody (anti SMT-succinyl-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)	sem		% cross reactivity
SMT	35.1	± 4.4	a	100
NA-SMT	15.6	± 1.5	bc	226
NP-SMT	9.20	± 2.70	c	382
succinyl-SMT	8.27	± 0.00	c	425
NAHis-azo-SMT	21.1	± 1.0	b	166
NCATyr-azo-SMT	21.4	± 1.6	b	164

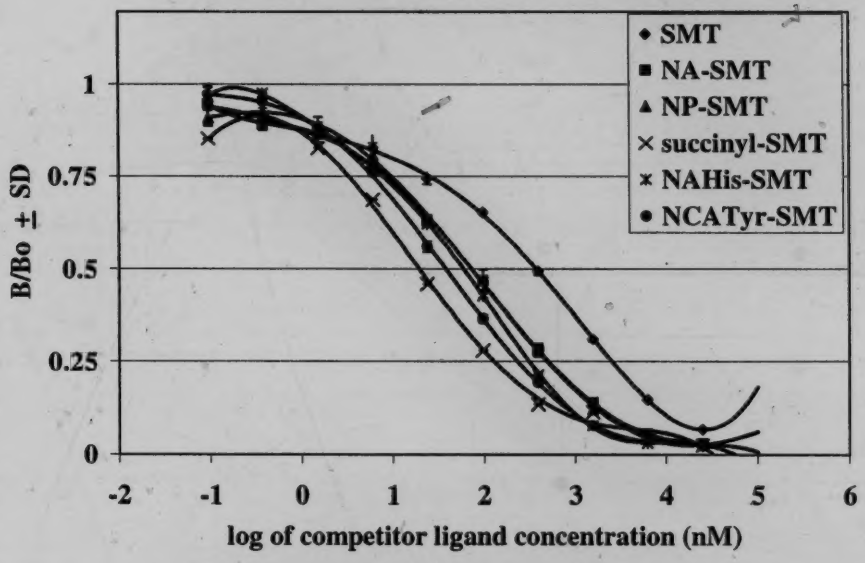
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.9. Competitive ELISA with Anti-SMT Serum 3B

Competitive ELISA functions for sulfamethazine ligands using 3B rabbit serum antibodies (anti-SMT-succinyl-LPH)



Sulfonamide derivatives in competitive ELISA using 3B serum antibody (anti SMT-succinyl-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)		sem		% cross reactivity
SMT	378	±	14	a	100
NA-SMT	73.3	±	5.0	b	516
NP-SMT	64.8	±	9.0	b	583
succinyl-SMT	18.0	±	-	c	2103
NAHis-azo-SMT	62.0	±	4.3	b	610
NCATyr-azo-SMT	44.8	±	2.9	bc	843

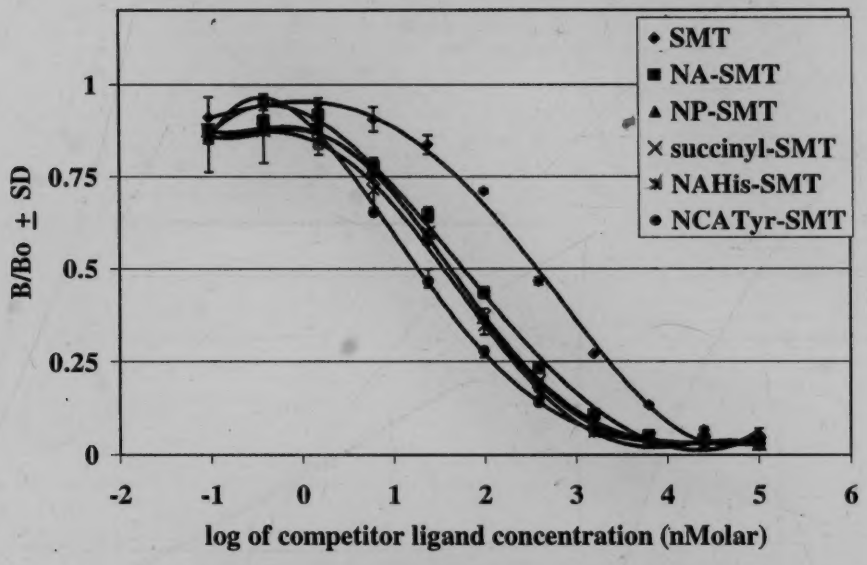
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.10. Competitive ELISA with Anti-SMT Immunopurified Antibody 3B

Competitive ELISA functions for sulfamethazine ligands using 3B rabbit immunopurified antibodies (anti-SMT-succinyl-LPH)



Sulfonamide derivatives in competitive ELISA using 3B immunopurified antibody (anti SMT-succinyl-LPH) and SMT-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)		sem		% cross reactivity
SMT	312	±	31	a	100
NA-SMT	53.9	±	6.9	b	579
NP-SMT	49.7	±	1.3	b	628
succinyl-SMT	51.7	±	2.1	b	604
NAHis-azo-SMT	49.9	±	5.8	b	625
NCATyr-azo-SMT	20.9	±	2.6	b	1494

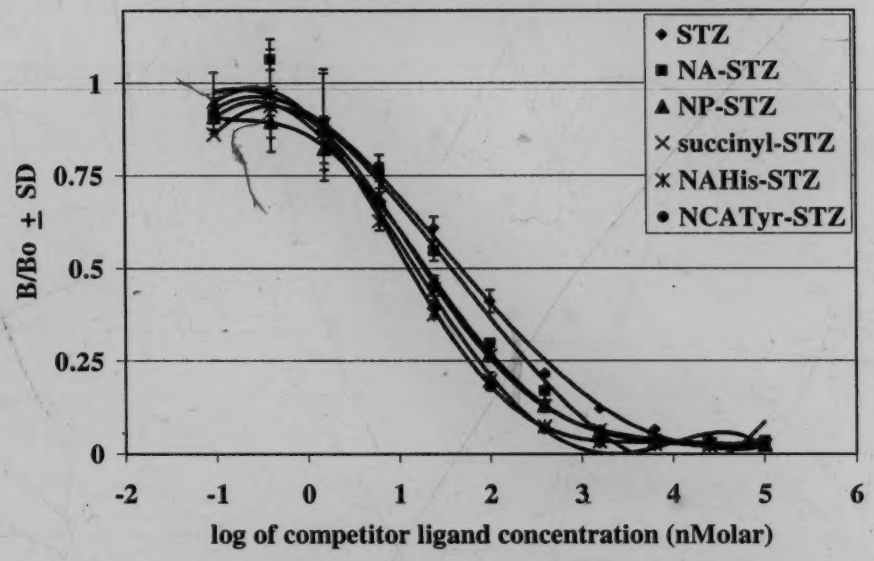
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.11. Competitive ELISA with Anti-STZ Serum 1C

Competitive ELISA functions for sulfathiazole ligands using 1C rabbit serum antibodies (anti-STZ-azo-LPH)



Sulfonamide derivatives in competitive ELISA using 1C serum antibody (anti STZ-azo-LPH) and STZ-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)	±	sem		% cross reactivity
STZ	111	±	17	a	100
NA-STZ	33.8	±	4.6	b	327
NP-STZ	21.7	±	2.9	b	509
succinyl-STZ	18.2	±	0.5	b	608
NAHis-azo-STZ	11.6	±	0.0	b	956
NCATyr-azo-STZ	12.2	±	0.4	b	902

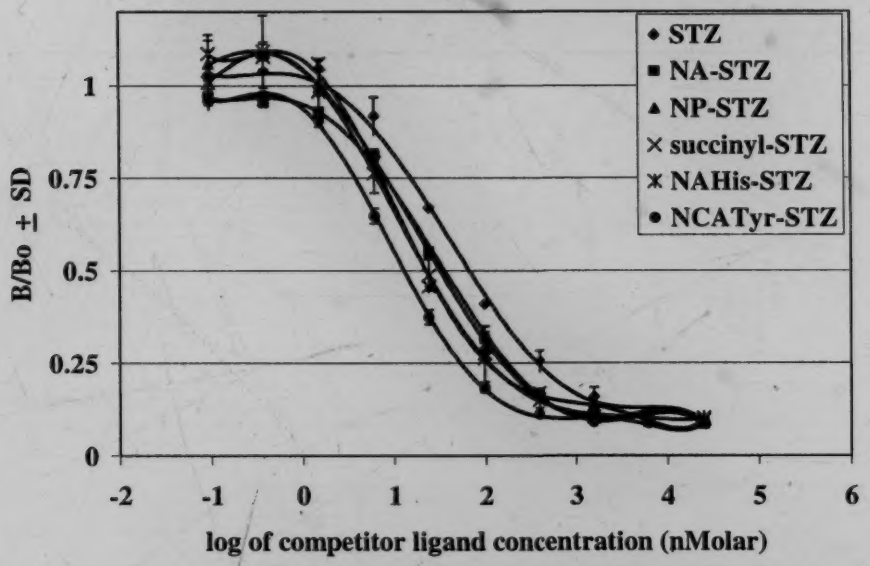
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.12. Competitive ELISA with Anti-STZ Immunopurified Antibody 1C

Competitive ELISA functions for sulfathiazole ligands using 1C rabbit immunopurified antibodies (anti-STZ-azo-LPH)



Sulfonamide derivatives in competitive ELISA using 1C immunopurified antibody (anti STZ-azo-LPH) and STZ-azo-BSA coating antigen

Competitor	IC_{50} (nM) Mean (n=2)		sem		% cross reactivity
STZ	56.1	±	0.5	a	100
NA-STZ	34.0	±	1.6	b	165
NP-STZ	27.7	±	0.4	c	203
succinyl-STZ	23.1	±	1.3	d	243
NAHis-azo-STZ	15.4	±	0.2	e	365
NCATyr-azo-STZ	12.7	±	1.3	e	441

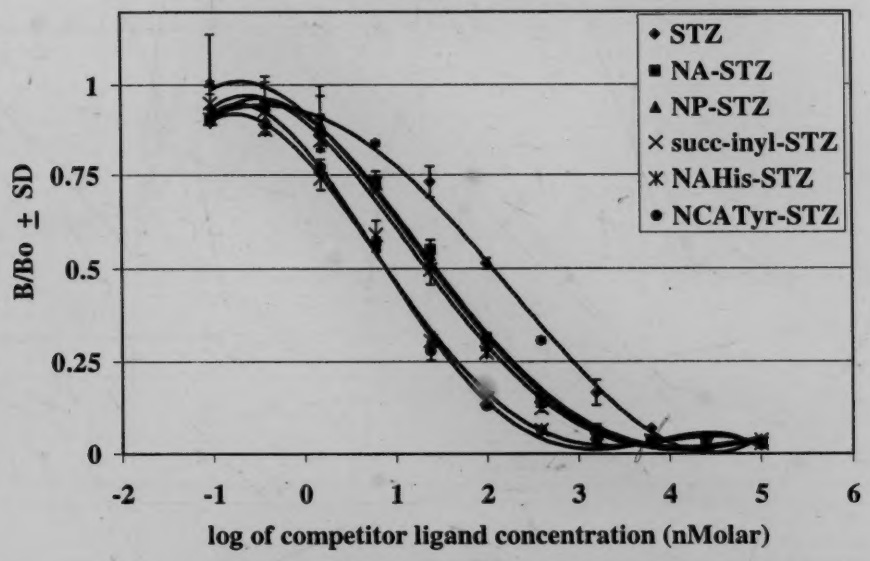
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different (p>0.05)

Appendix 9.4.13. Competitive ELISA with Anti-STZ Serum 1D

Competitive ELISA functions for sulfathiazole ligands using 1D rabbit serum antibodies (anti-STZ-azo-LPH)



Sulfonamide derivatives in competitive ELISA using 1D serum antibody (anti STZ-azo-LPH) and STZ-azo-BSA coating antigen

Competitor	IC_{50} (nM) average (n=2)		sem		% cross reactivity
STZ	108	±	5	a	100
NA-STZ	30.8	±	5.8	b	350
NP-STZ	28.2	±	3.5	b	381
succinyl-STZ	25.7	±	2.3	b	419
NAHis-azo-STZ	9.57	±	1.22	c	1125
NCATyr-azo-STZ	8.29	±	0.57	c	1299

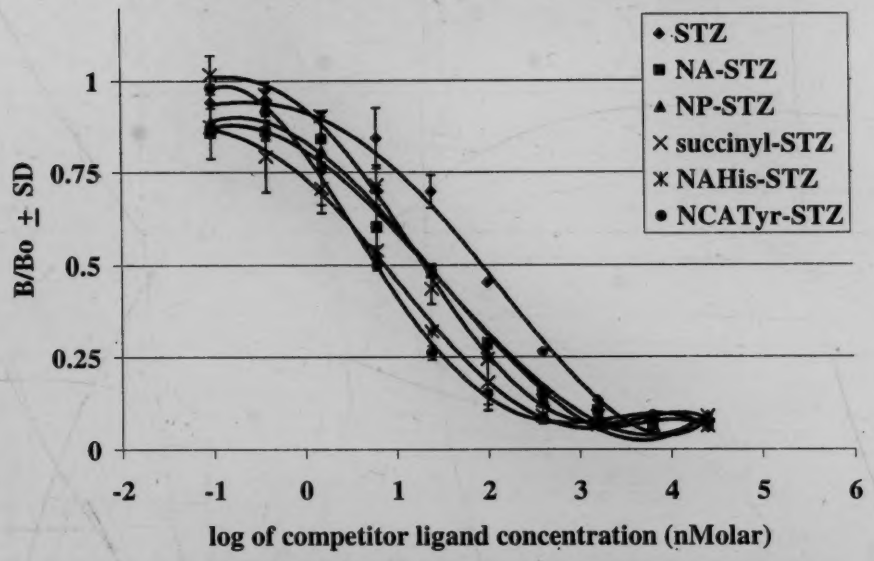
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.14. Competitive ELISA with Anti-STZ Immunopurified Antibody 1D

Competitive ELISA functions for sulfathiazole ligands using 1D rabbit immunopurified antibodies (anti-STZ-azo-LPH)



Sulfonamide derivatives in competitive ELISA using 1D immunopurified antibody (anti STZ-azo-LPH) and STZ-azo-BSA coating antigen

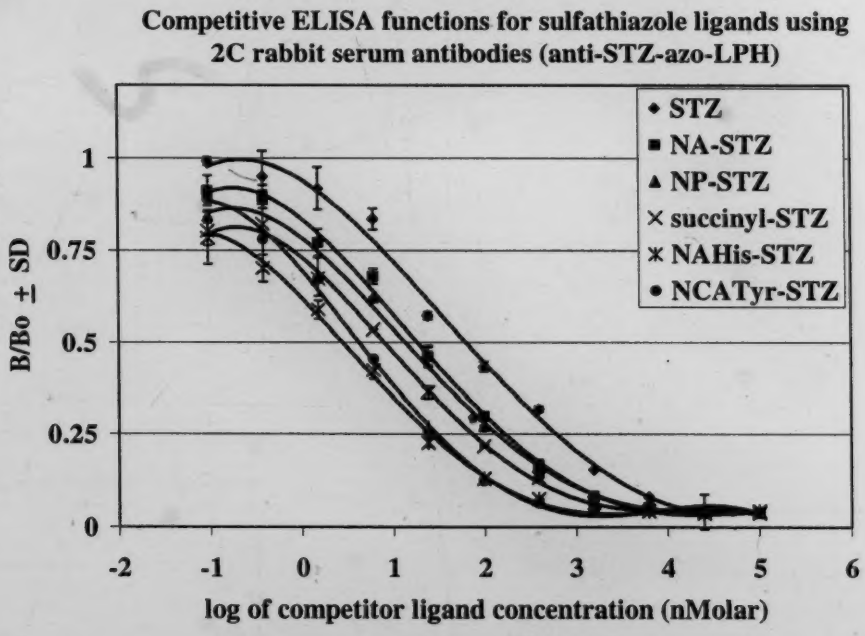
Competitor	IC_{50} (nM) average (n=2)		sem		% cross reactivity
STZ	79.0	±	2.1	a	100
NA-STZ	20.5	±	0.8	bc	385
NP-STZ	23.4	±	1.2	b	338
succinyl-STZ	17.2	±	2.2	c	459
NAHis-azo-STZ	8.34	±	0.14	d	947
NCATyr-azo-STZ	4.88	±	0.59	d	1618

IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.15. Competitive ELISA with Anti-STZ Serum 2C



Sulfonamide derivatives in competitive ELISA using 2C serum antibody (anti STZ-azo-LPH) and STZ-azo-BSA coating antigen

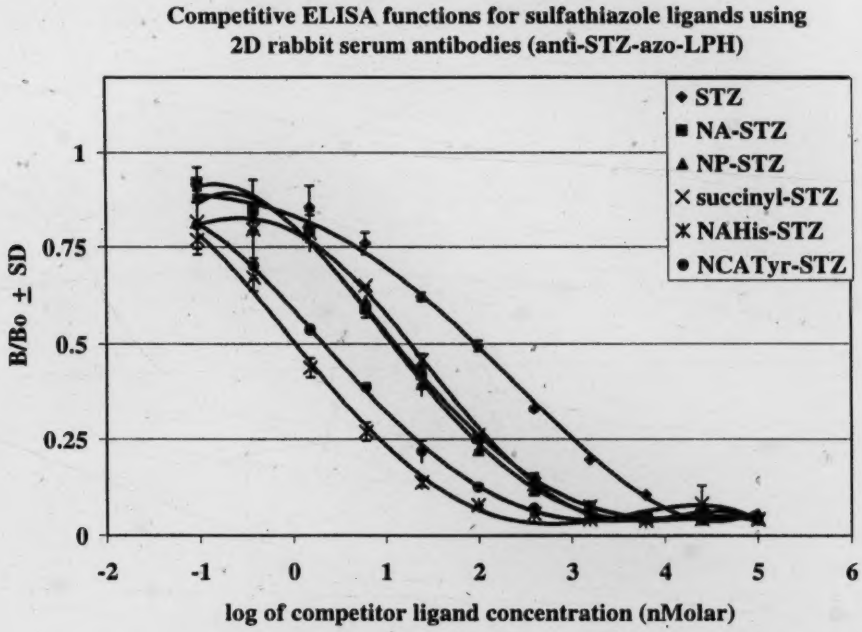
Competitor	IC_{50} (nM) average (n=2)		sem		% cross reactivity
STZ	53.2	±	13.5	a	100
NA-STZ	24.0	±	4.9	b	222
NP-STZ	16.3	±	0.7	bc	327
succinyl-STZ	8.98	±	0.96	cd	592
NAHis-azo-STZ	2.48	±	0.38	d	2143
NCATyr-azo-STZ	4.44	±	0.28	d	1197

IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.16. Competitive ELISA with Anti-STZ Serum 2D



Sulfonamide derivatives in competitive ELISA using 2D serum antibody (anti STZ-azo-LPH) and STZ-azo-BSA coating antigen

Competitor	IC_{50} (nM) average (n=2)		sem		% cross reactivity
STZ	52.8	±	1.8	a	100
NA-STZ	11.6	±	1.5	b	455
NP-STZ	13.2	±	0.2	b	402
succinyl-STZ	13.5	±	4.9	b	390
NAHis-azo-STZ	1.32	±	0.02	c	4001
NCATyr-azo-STZ	2.86	±	0.19	bc	1848

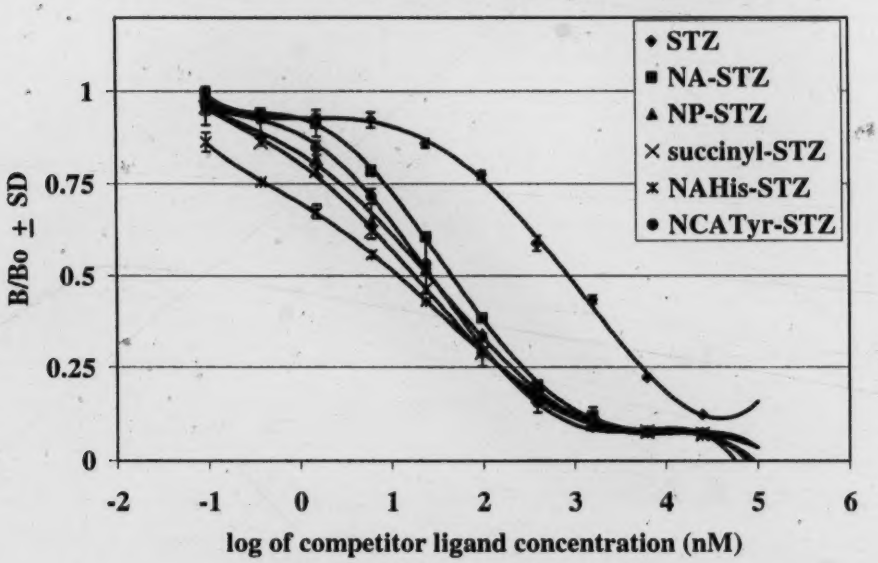
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different (p>0.05)

Appendix 9.4.17. Competitive ELISA with Anti-STZ Serum 3A

Competitive ELISA functions for sulfathiazole ligands using 3A rabbit serum antibodies (anti-STZ-succinyl-LPH)



Sulfonamide derivatives in competitive ELISA using 3A serum antibody (anti STZ-succinyl-LPH) and STZ-azo-BSA coating antigen

Competitor	IC_{50} (nM) average (n=2)		sem		% cross reactivity
STZ	846	±	63	a	100
NA-STZ	43.7	±	0.5	b	1934
NP-STZ	25.0	±	3.2	b	3381
succinyl-STZ	15.5	±	0.1	b	5449
NAHis-azo-STZ	11.9	±	0.8	b	7098
NCATyr-azo-STZ	31.6	±	8.4	b	2675

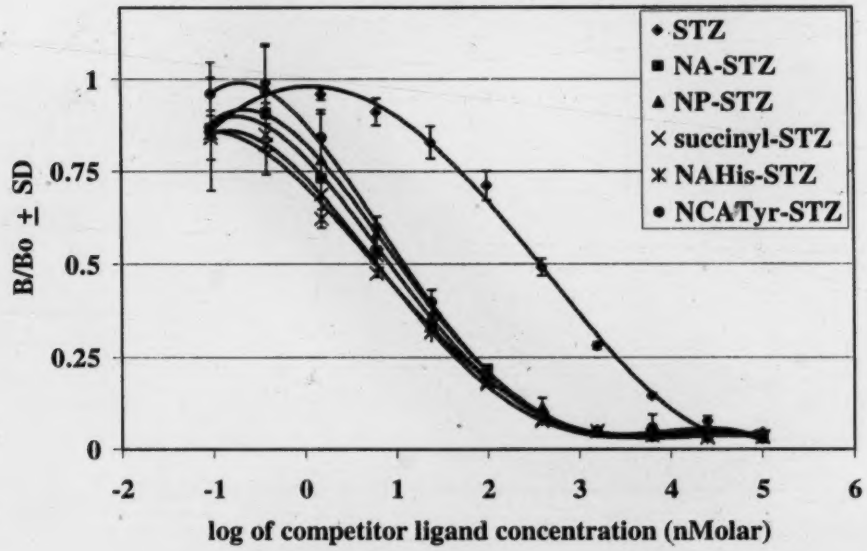
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.18. Competitive ELISA with Anti-STZ Immunopurified Antibody 3A

Competitive ELISA functions for sulfathiazole ligands using 3A rabbit immunopurified antibodies (anti-STZ-succinyl-LPH)



Sulfonamide derivatives in competitive ELISA using 3A immunopurified antibody (anti STZ-succinyl-LPH) and STZ-azo-BSA coating antigen

Competitor	IC_{50} (nM) average (n=2)		sem		% cross reactivity
STZ	902	±	106	a	100
NA-STZ	48.6	±	8.6	b	1854
NP-STZ	37.5	±	4.1	b	2403
succinyl-STZ	13.4	±	2.2	b	6716
NAHis-azo-STZ	22.7	±	7.1	b	3971
NCATyr-azo-STZ	28.2	±	0.2	b	3198

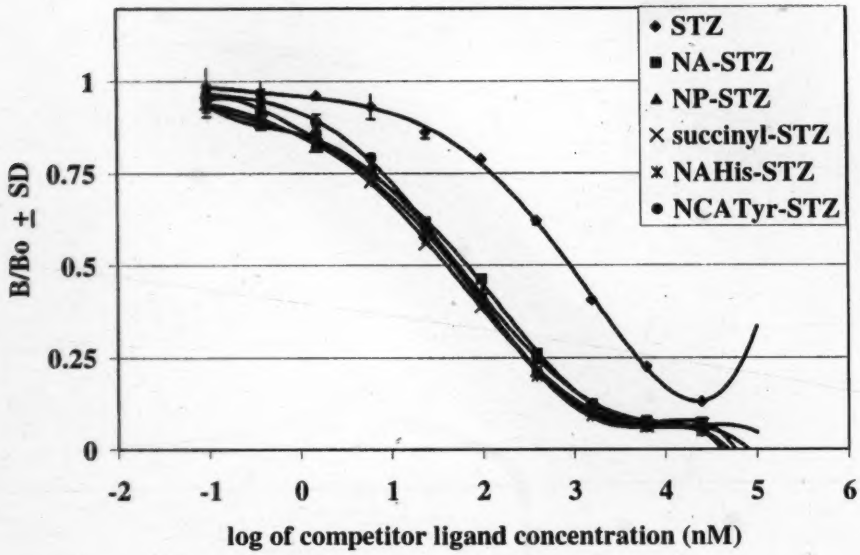
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.19. Competitive ELISA with Anti-STZ Serum 3B

Competitive ELISA functions for sulfathiazole ligands using 3B rabbit serum antibodies (anti-STZ-succinyl-LPH)



Sulfonamide derivatives in competitive ELISA using 3B serum antibody (anti STZ-succinyl-LPH) and STZ-azo-BSA coating antigen

Competitor	IC_{50} (nM) average (n=2)		sem		% cross reactivity
STZ	892	±	53	a	100
NA-STZ	73.5	±	12.1	b	1214
NP-STZ	52.8	±	7.5	b	1691
succinyl-STZ	44.9	±	4.9	b	1987
NAHis-azo-STZ	50.5	±	2.9	b	1765
NCATyr-azo-STZ	52.5	±	0.5	b	1698

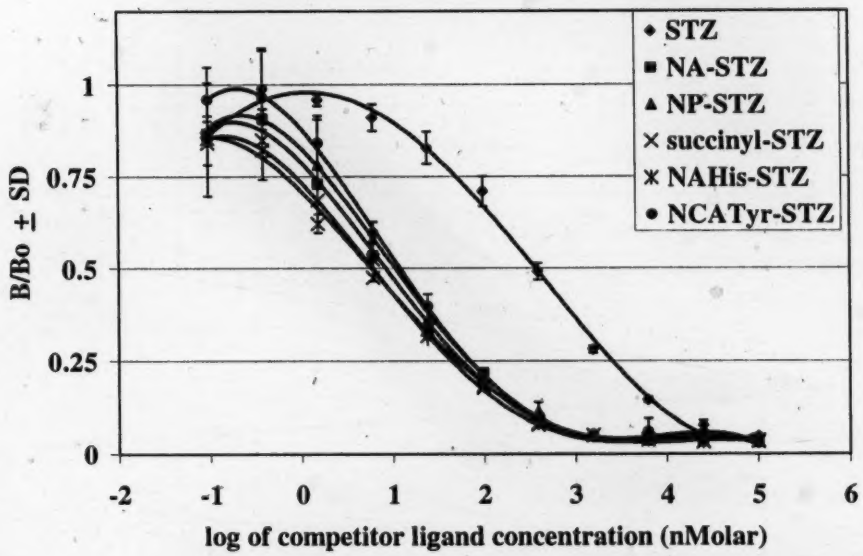
IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)

Appendix 9.4.20. Competitive ELISA with Anti-STZ Immunopurified Antibody 3B

Competitive ELISA functions for sulfathizole ligands using 3B rabbit immunopurified antibodies (anti-STZ-succinyl-LPH)



Sulfonamide derivatives in competitive ELISA using 3B immunopurified antibody (anti STZ-succinyl-LPH) and STZ-azo-BSA coating antigen

Competitor	IC_{50} (nM) average (n=2)		sem		% cross reactivity
STZ	359	±	33	a	100
NA-STZ	7.12	±	0.83	b	5041
NP-STZ	10.2	±	2.0	b	3512
succinyl-STZ	6.35	±	0.69	b	5656
NAHis-azo-STZ	5.52	±	0.92	b	6501
NCATyr-azo-STZ	11.3	±	1.4	b	3179

IC_{50} = [sulfonamide] (nM) required to inhibit antibody binding by 50%

% cross reactivity = 100*IC_{50} parent sulfonamide / IC_{50} competitor

Means with the same letters are not significantly different ($p>0.05$)